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Toxicological Testing of Organic Substances from Concentrated Drinking and Waste Waters

Environmental Health Research and Testing, Inc.
Research Triangle Park, NC

Prepared for

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16. ABSTRACT

The U.S. Environmental Protection Agency has been given the responsibility for regulating the release of toxic chemicals into the environment. Accordingly, the EPA presently conducts an extensive and comprehensive research program to determine the adverse effects of environmental factors on human health. A considerable amount of this research activity is directed toward toxicological testing and test development. The Health Effects Research Laboratory of the EPA, in Cincinnati, Ohio, has specifically focussed on the task of determining, through the use of appropriate short-term assays, the potential health hazards of the complex mixtures of organic compounds found in drinking and waste waters. A battery testing approach is being employed by the EPA at Cincinnati for the toxicological testing of these waters, progressing from routine detection systems in microbial cells to more complex systems in mammalian cells and subsequently to short-term animal testing.

In support of the EPA's inhouse research and testing, a study was conducted employing a battery of five short-term assays for the toxicological testing of coded concentrated drinking and waste water samples. The five assays performed were: (1) the *in vivo* toxicity test, (2) *Salmonella typhimurium* mutagenesis assay, (3) Sencar mouse skin bioassay, (4) rat liver focus assay, and (5) lung adenoma assay. The Final Report for this project gives the results of the testing of a total of 40 coded water samples in one or more of the above assays.

This project summary was submitted in fulfillment of Contract No. 68-03-1840 by Environmental Health Research and Testing, Inc. under the sponsorship of the U.S. Environmental Protection Agency. This summary covers a period from February 1984 to September 30, 1985, and work was completed as of September 30, 1985.

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by

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68-03-1840

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NOTICE

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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The complexities of environmental problems originate in the deep interdependent relationships between the various physical and biological segments of man's natural and social world. Solutions to these environmental problems require an integrated program of research and development using input from a number of disciplines. The Health Effects Research Laboratory, Research Triangle Park, NC and Cincinnati, OH, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. Wide ranges of pollutants known or suspected to cause health problems are studied. The research focuses on air pollutants, water pollutants, toxic substances, hazardous wastes, pesticides, and non-ionizing radiation. The laboratory participates in the development and revision of air and water quality criteria and health assessment documents on pollutants for which regulatory actions are being considered. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of environmental regulatory decisions involving the protection of the health and welfare of all U.S. inhabitants.

One specific task undertaken by the Health Effects Research Laboratory of the EPA, in Cincinnati, Ohio, has been that of determining, through the use of appropriate short-term assays, the potential health hazards of the complex mixtures of organic compounds found in drinking and waste waters. A battery testing approach is being employed for the toxicological testing of these waters, progressing from routine detection systems in microbial cells to more complex systems in mammalian cells and subsequently to short-term animal testing. In support of the EPA's inhouse research and testing, a study was conducted employing a battery of five short-term assays for the toxicological testing of coded concentrated drinking and waste water samples. The five assays performed were: (1) the *in vivo* toxicity test, (2) *Salmonella typhimurium* mutagenesis assay, (3) Sencar mouse skin bioassay, (4) rat liver focus assay, and (5) lung adenoma assay. This document gives the results of the testing of a total of 40 coded water samples in one or more of the above assays.

F. Gordon Hueter, Ph.D.
Director
Health Effects Research Laboratory

PREFACE

At the conclusion of this study, the EPA provided to EHRT, for inclusion in the Final Report, a schematic of the Jefferson Parish, La. pilot plant where the sampling was done and source identifications for all coded samples tested. This information is included herein as Figure 1 and Tables 1, 2, 3 and 4.

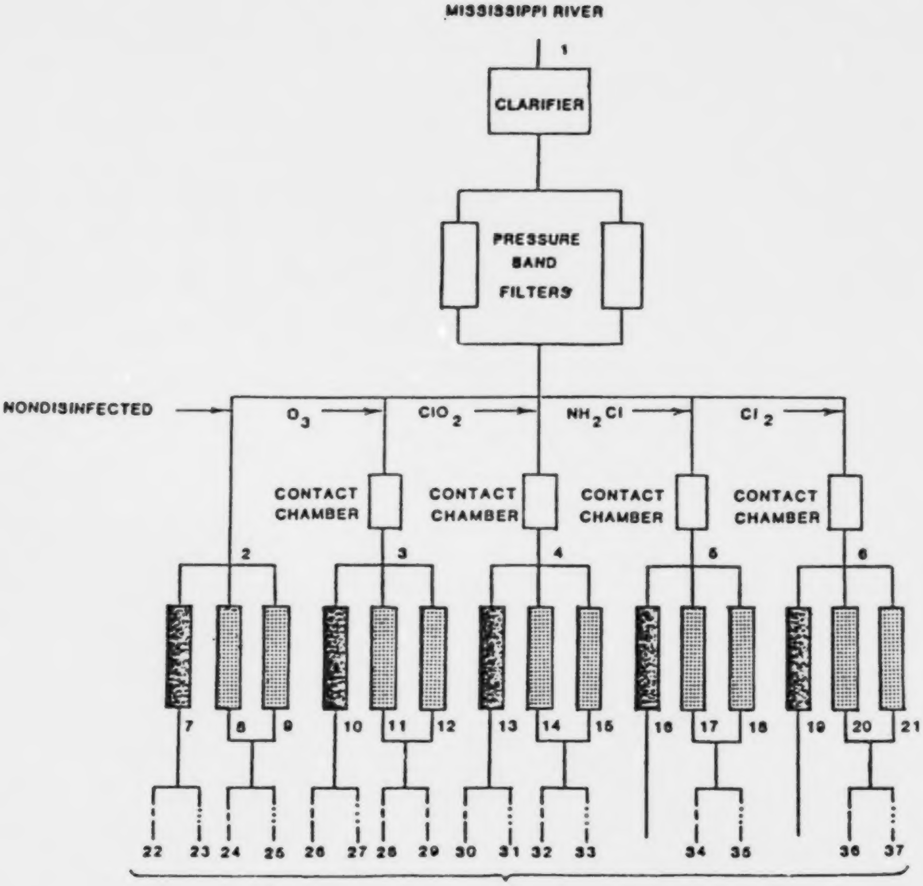
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Figure 1. Flow Schematic of Jefferson Parish, La. Pilot Plant

Table 1. Sample and Source Identification for the Eight Sample Series

Sample Number[1]	Sample Identification	Sample Source[2]
1-6L-P74	Nondisinfectant Stream	J.P. Sample Point #2
2-6L-P76	Ozone Disinfectant Stream	J.P. Sample Point #3
3-6L-P78	Chlorine Dioxide Disinfectant Stream	J.P. Sample Point #4
4-6L-P80	Monochloramine Disinfectant Stream	J.P. Sample Point #5
5-6L-P82	Chlorine Disinfectant Stream	J.P. Sample Point #6
6A-2G-A-1+A-2-P70	Primary Waste Effluent concentrated 10X by reverse osmosis - sterile filtered	EPA Test & Evaluation Gest St., Cinti., OH
6B-2G-B-1+B-2-P68	Primary Waste Effluent non concentrated - sterile filtered	EPA Test & Evaluation Gest St., Cinti., OH
7A-2G-A-1+A-2-P66	Clarified Waste Effluent concentrated 10X by reverse osmosis - sterile filtered	EPA Test & Evaluation Gest St., Cinti., OH
7B-2G-B-1+B-2-P64	Clarified Waste Effluent non concentrated - sterile filtered.	EPA Test & Evaluation Gest St., Cinti., OH
8A-2G-A-1+A-2-P62	Clarified and Chlorinated Effluent concentrated 10X by reverse osmosis - sterile filtered	EPA Test & Evaluation Gest St., Cinti., OH
8B-2G-B-1+B-2-P60	Clarified and Chlorinated Effluent non concentrated - sterile filtered	EPA Test & Evaluation Gest St., Cinti., OH

[1] Samples #1-5 represent 1000X concentrated drinking water samples prior to contractors additional concentrating.

[2] J.P. = Jefferson Parish Pilot Plant

Table 2. Sample and Source Identification for the Nine Sample Series

Sample No.[1]	Sample Identification	Sample Source[2]
1-5L-P86	Nondisinfectant Stream	J.P. Sample Point #2
2-5L-P88	Ozone Disinfectant Stream	J.P. Sample Point #3
3-5L-P90	Chlorine Dioxide Disinfectant Stream	J.P. Sample Point #4
4-5L-P92	Monochloramine Disinfectant Stream	J.P. Sample Point #5
5-5L-P94	Chlorine Disinfectant Stream	J.P. Sample Point #6
6-5L-P96	Chlorine Stream After GAC	J.P. Sample Point #20 & #21
7-5L-P98	Chlorine Stream + GAC Rechlorinated	J.P. Sample Point #36
8-5L-P30	Nondisinfectant Stream	J.P. Sample Point #2
9-5L-P38	Chlorine Disinfectant Stream	J.P. Sample Point #6

[1] These samples represent 1000X concentrated drinking water samples prior to contractors additional concentrating.

[2] J.P. = Jefferson Parish Pilot Plant.

Table 3. Sample and Source Identification for the Eighteen Sample Series

Sample No.[1]	Sample Identification	Sample Source
10-80-P32	Ozone Disinfectant Stream	J.P. Sample Point #3
11-80-P34	Chlorine Dioxide Disinfectant Stream	J.P. Sample Point #4
12-80-P36	Monochloramine Disinfectant Stream	J.P. Sample Point #5
13-80-P40	Monochloramine Disinfectant Stream After GAC	J.P. Sample Point #17 & #18
14-80-P42	Monochloramine Disinfectant Stream After GAC Plus Monochloramine	J.P. Sample Point #35
15-80-P14	Nondisinfectant Stream	J.P. Sample Point #2
16-80-P16	Ozone Disinfectant Stream	J.P. Sample Point #3
17-80-P18	Chlorine Dioxide Disinfectant Stream	J.P. Sample Point #4
18-80-P20	Monochloramine Disinfectant Stream	J.P. Sample Point #5
19-80-P22	Chlorine Disinfectant Stream	J.P. Sample Point #6
20-80-P24	Chlorine Disinfectant Stream After GAC	J.P. Sample Point #20 & #21
21-80-P26	Chlorine Disinfectant Stream + GAC Rechlorinated	J.P. Sample Point #36
22-80-P46	Clarified Waste Effluent - Concentrated 275X by XAD Resins	EPA Test & Evaluation Gest St., Cinti., OH
23-80-P48	Clarified Waste Effluent - Concentrated 275X by XAD Resins	EPA Test & Evaluation Gest St., Cinti., OH
24-80-P50	Clarified and Chlorinated Waste Effluent - Concentrated 275X by XAD Resins	EPA Test & Evaluation Gest St., Cinti., OH
25-80-P52	Clarified and Chlorinated Waste Effluent - Concentrated 275X by XAD Resins	EPA Test & Evaluation Gest St., Cinti., OH
26-80-P54	Primary Waste Effluent - Concentrated 275X by XAD Resins	EPA Test & Evaluation Gest St., Cinti., OH
27-80-P56	Primary Waste Effluent - Concentrated 275X by XAD Resins	EPA Test & Evaluation Gest St., Cinti, OH

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[1] Samples #10-21 represent 1000X concentrated drinking water samples prior to contractors additional concentrating.

Table 4. Sample and Source Identification for the Five Sample Series

Sample No.	Sample Identification	Sample Source[1]
1-9/84	Nondisinfectant Stream Concentrated 4000X	J.P. Sample Point #2
2-9/84	Monochloramine Disinfectant Stream Concentrated 4000X	J.P. Sample Point #5
3-9/84	Chlorine Disinfectant Stream Concentrated 4000X	J.P. Sample Point #6
4-9/84	Chlorine Disinfectant Stream GAC Concentrated 4000X	J.P. Sample Point #20 & #21
5-9/84	Chlorine Stream After GAC Plus Chlorine Concentrated 4000X	J.P. Sample Point #36

[1] J.P. = Jefferson Parish Pilot Plant

ABSTRACT

A battery of five short-term bioassays were employed for the toxicological testing of concentrated drinking and waste water samples. These assays were: (1) the *in vivo* toxicity test in mice, (2) *Salmonella typhimurium* mutagenesis assay, (3) Sencar mouse skin bioassay: initiation/promotion, (4) rat liver focus assay and (5) lung adenoma assay in Strain A mice. Four coded sample groups were tested: (1) the Eight Sample Series, (2) the Nine Sample Series, (3) the Eighteen Sample Series, and (4) the Five Sample Series. Each sample series was tested in one or more assays.

The Eight Sample Series was tested only in the *in vivo* toxicity test (Assay 1); all samples were negative.

The Nine Sample Series was tested in Assays 2, 3, 4, and 5. Positive results were observed only in Assay 2, with five of the nine samples showing a positive response at one or more sample doses. Experiments performed during the course of the study indicated that there were no significant differences in mutagenic response between samples concentrated and stored in 2% Emulphor at 4°C for 62 weeks, for 33 weeks, for 11 weeks, for 3 weeks, and for 2 weeks, i.e. the relative mutagenic potency of the nine samples remained consistent over the course of study. In an experiment comparing the stability of samples stored in 2% Emulphor for 11 weeks at 4°C and those freshly concentrated from original acetone samples stored at -20°C for the same period, similar mutagenic responses were observed, thus indicating that the mutagenic compounds in these nine samples were stable under two different storage conditions. It was also observed that the presence of the S9 activation system decreased or eliminated the positive responses observed without S9 addition.

The Eighteen Sample Series was tested only in Assay 2, with mutagenic activity evaluated in two separate experiments. Results indicated that the samples positive in the first experiment lost mutagenic activity over the 34 week storage period prior to retest, with a decrease from 7 to 4 positive samples for TA98 and from 5 to 4 for TA100, even though the sample dose range was increased in the retest. Ten samples were negative in both experiments. Except for one sample, it was again observed that the presence of an S9 activating system decreased the positive responses.

The Five Sample Series was tested in Assays 1, 2, 4 and 5. Positive results were observed only in Assay 2, with three of the five samples showing a positive mutagenic response at one or more sample doses. For all three positive samples, the presence of the S9 activating system decreased or negated the positive responses observed without S9 addition.

This report was submitted in fulfillment of Contract No. 68-03-1840 by Environmental Health Research and Testing, Inc. under the sponsorship of the U.S. Environmental Protection Agency. This report covers a period from February 1984 to September 30, 1985, and work was completed as of September 30, 1985.

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SECTION 1

INTRODUCTION

The U.S. Environmental Protection Agency (EPA) has been given the responsibility for regulating the release of toxic chemicals into the environment under the Toxic Substances Control Act, Resource Conservation and Recovery Act, Federal Insecticide, Fungicide and Rodenticide Act, and the Clean Air and Water Acts. Along with the regulatory aspects of the above Acts, the EPA conducts an extensive and comprehensive research program to determine the adverse effects of environmental factors on human health. The purpose of these research programs is to provide information that is essential to formulate regulatory and compliance policies to protect public health and the environment.

With the advent of the Toxic Substances Control Act and evidence showing the relationship between environmental agents and adverse health effects (cancer, toxicity, developmental and genetic defects, pulmonary disease, etc.), the EPA has directed a considerable amount of activity into toxicological testing and test development. Due to the large number of potentially hazardous chemicals being introduced each year into the environment, the backlog of untested chemicals already present, and the prohibitive cost and time necessary to assess chronic effects of these agents in long-term animal testing, the EPA has been particularly interested in the development, utilization, and evaluation of time- and cost-effective short-term *in vivo* and *in vitro* test systems.

In accordance with the above objectives, the Health Effects Research Laboratory of the EPA, in Cincinnati, Ohio, has specifically focussed on the task of determining, through the use of appropriate short-term assays, the potential health hazards of the complex mixtures of organic compounds found in drinking and waste waters. Preparation of these mixtures in sufficient quantity for toxicological testing has been made possible by the development of concentration (reverse osmosis) and isolation (using various macroreticular resins) techniques that concentrate water and/or waste water contaminants by 400- to 1000-fold. The identification, characterization, and possible interaction of these materials presents a formidable problem. Moreover, it is recognized that man is exposed to these compounds as complex mixtures and thus, that risk to man associated with these exposures can best be determined by toxicological testing in animals or *in vitro* testing at biological levels of organization that may simulate the response in man.

It has been proposed that testing of environmental agents proceed through a battery of well-defined and controlled short-term *in vivo* and in vitro assays that are reproducible between laboratories, possess a high degree of correlation with the chronic in vivo effects of various chemical compounds, and provide information relevant to effects observed in man. Ideally, these assays should generate no false-negative results and preferably only a low percentage of false-positives, be responsive to diverse classes of chemical agents, and lend themselves to quantitation. In addition, the assays should be relatively inexpensive, rapid and technically simple. Such a battery approach is presently being employed by the EPA at Cincinnati for the toxicological testing of drinking/waste waters progressing from routine detection systems in microbial cells to more complex systems in mammalian cells and subsequently to short-term animal testing.

In support of the EPA's inhouse research and testing, EHRT has under Contract No. 68-03-1840, employed a battery of five short-term assays for the "Toxicological Testing of Organic Substances from Concentrated Drinking and Waste Waters". The five assays performed were: (1) the in vivo toxicity test in mice, (2) Salmonella typhimurium mutagenesis assay, (3) Sencar mouse skin bioassay: initiation/promotion, (4) rat liver focus assay and (5) lung adenoma assay in Strain A mice.

The in vivo toxicity tests (Assay #1) and lung adenoma assays (Assay #5) were performed at EHRT's Cincinnati laboratory, initially under the direction of Dr. H. Choudhury and later under the direction of Dr. R. York. The histopathology requirements of the lung adenoma assays were carried out by Pathology Associates, Inc. (PAI).

The Salmonella mutagenesis assays (Assay #2) were directed by Ms. L. Snow at EHRT's Research Triangle Park (RTP) location and performed by Mr. T. Hughes and his staff at Research Triangle Institute (RTI), RTP, NC.

The Sencar mouse skin bioassays (Assay #3) and the rat liver focus assays (Assay #4) were performed by Microbiological Associates, Inc. (MAI), under subcontract to EHRT, and were directed by MAI's Dr. M. Wenk. The histopathology requirements of both assays were carried out by PAI.

SECTIONS 2 and 3

CONCLUSIONS AND RECOMMENDATIONS

Four coded groups of concentrated drinking or waste water samples, identified as the Eight Sample Series, the Nine Sample Series, the Eighteen Sample Series, and the Five Sample Series, were screened for toxicological activity in one or more of the following bioassays: (1) *in vivo* toxicity test, (2) *Salmonella typhimurium* mutagenesis assay, (3) Sencar mouse skin bioassay, (4) rat liver focus assay, and (5) lung adenoma assay.

Positive results were obtained with the *Salmonella* screen (strains TA100 and TA98). Several mutagenic samples were present in each of the three sample groups - Nine, Eighteen, and Five Sample Series - tested with this assay. However, it was observed that the relative activity of these samples, at the concentrations tested, was somewhat low as compared to most known mutagenic compounds. It was also observed that the presence of the S9 activation system decreased or eliminated the positive responses observed without S9 addition. Experimental results indicated that some of the concentrated water samples lost activity during storage at 4°C in the Emulphor vehicle (Eighteen Sample Series) while other samples retained their activity (Nine Sample Series). It was also determined that the mutagenic compounds in the Nine Sample Series were stable under different storage conditions i.e. concentrated and stored in 2% Emulphor for 11 weeks at 4°C versus freshly concentrated from original acetone samples stored at -20°C for the same period.

No carcinogenic activity was observed with any of the coded samples tested in the Sencar, rat liver focus, or lung adenoma assays, nor was any toxic activity observed with coded samples tested in the *in vivo* toxicity test. The weak activity of samples positive in the *Salmonella* assay suggests that the concentrations of mutagens/carcinogens in the samples might have been too low for detection in the animal bioassays. However, in view of the toxic responses observed in the rat liver focus assay, additional concentration, using the same methods, might not be productive. Rather, it might be necessary to fractionate the sample concentrates, in an attempt to separate the toxic components of these complex mixtures from their mutagenic/carcinogenic components. Sensitive short-term tests such as the *Salmonella* screen might be useful in guiding these fractionation procedures.

In lieu of fractionation and additional concentration, potentially more sensitive *in vitro* and *in vivo* systems may be employed for identifying the toxic/mutagenic/carcinogenic compounds in water samples.

Such systems for detecting cell transformation *in vitro* include: the hamster embryo focus or colony assay, the mouse fibroblast C3H10T1/2 CL8 assay, and the Syrian hamster embryo/SA7 enhancement assay. For measurement of toxic activity *in vitro*, inhibition of growth of pig aortic endothelial cells may be used. Some *in vivo* systems, which may prove to be more sensitive for measurement of activity in concentrated drinking and waste waters, include: the sex-linked recessive lethal test in *Drosophila*, the detection of sister chromatid exchanges (SCE's) in the liver, lung or other major organs of mice, and the rat neonatal survival assay.

The inclusion of this latter *in vivo* assay is particularly important, since developmental toxicity can result from nonmutagenic/noncarcinogenic mechanisms. Moreover, the test parameters in the rat neonatal survival assay are not limited to the assessment of gross abnormalities in structural development but also include endpoints such as adult fertility, stillbirths, pre- and post-natal growth retardation and functional deficits in the offspring.

SECTION 4

MATERIALS AND METHODS

4.1 SAMPLES TESTED

All samples tested by EHRT under EPA contract 68-03-1840 and the assay systems in which they were tested are shown in Table 4-1.

4.2 *IN VIVO* TOXICITY TEST IN MICE

4.2.1 *Source of Animals*

Six-week old virus and parasite free CD-1 mice (male and female) were purchased from Harlan Industries, Indiana.

4.2.2 *Receipt of Animals*

Upon receipt, all animals were inspected, weighed, placed in numbered cages using a table of random numbers, and permanently identified with an ear punch number on the left ear.

4.2.3 *Quarantine Procedures*

During a subsequent two-week quarantine period, randomly selected male and female mice were checked for ectoparasites and for antibody titers against eleven known murine viruses. All results were negative. Cage-side observations of all mice were performed twice daily to check for fighting wounds and any unusual health-related conditions. In addition, all animals were closely examined at the time of weekly cage and bedding changes. On the last day of the quarantine period (the day prior to initiation of exposure to test material), the Staff Veterinarian reviewed the accumulated health data and conducted physical examinations of all mice. All animals judged healthy and suitable as test animals were released from quarantine.

4.2.4 *Animal Care*

For all tests, cages of mice were randomly assigned to test groups. Animals retained their permanent ear punch numbers given upon arrival and the cages labelled with those numbers. Males or females, five each per cage, were housed in separate plastic 10 1/2" x 8 1/8" cages placed on steel racks. Cages and bedding were changed weekly. Clean bottles containing deionized water or test (water) samples were given to all mice at least once a week. Purina Certified Rodent Chow was used as the basal

Table 4-1 Samples Tested

Date Received	Sample ID	Sample Volume	Tested In[b] Assay System
1/19/84	1-6L-P74	6 liters	1
	2-6L-P76	"	1
	3-6L-P78	"	1
	4-6L-P80	"	1
	5-6L-P82	"	1
	6A-2G-A-1+A-2-P70	2 gallons	1
	6B-2G-B-1+B-2-P68[a]	"	1
	7A-2G-A-1+A-2-P66	"	1
	7B-2G-B-1+B-2-P64[a]	"	1
	8A-2G-A-1+A-2-P62	"	1
	8B-2G-B-1+B-2-P60[a]	"	1
1/19/84	1-5L-P96	5 liters	2,3,4,5
	2-5L-P88	"	2,3,4,5
	3-5L-P90	"	2,3,4,5
	4-5L-P92	"	2,3,4,5
	5-5L-P94	"	2,3,4,5
	6-5L-P96	"	2,3,4,5
	7-5L-P98	"	2,3,4,5
	8-5L-P30	"	2,3,4,5
	9-5L-P38	"	2,3,4,5
1/19/84	10-80-P32	80 milliliters	2
	11-80-P34	"	2
	12-80-P36	"	2
	13-80-P40	"	2
	14-80-P42	"	2
	15-80-P14	"	2
	16-80-P16	"	2
	17-80-P18	"	2
	18-80-P20	"	2
	19-80-P22	"	2
	20-80-P24	"	2
	21-80-P26	"	2
	22-80-P46	"	2
	23-80-P48	"	2
	24-80-P50	"	2
	25-80-P52	"	2
	26-80-P54	"	2
	27-80-P56	"	2
9/24/84	1-9/84	1 liter	1,2,4,5
	2-9/84	"	1,2,4,5
	3-9/84	"	1,2,4,5
	4-9/84	"	1,2,4,5
	5-9/84	"	1,2,4,5

[a] Sample B is a 1:10 dilution of sample A above

[b] 1 = *In vivo* toxicity test

2 = *Salmonella* mutagenesis assay

3 = Sencar mouse skin bioassay

4 = Rat liver focus assay

5 = Lung adenoma assay

diet. Food and water were offered *ad libitum*. Temperature in the animal room was held at 20°C ± 5% and humidity at 50% ± 10%. Light was controlled automatically by a timer yielding 12 hr cycles of light-darkness. Air entering the room was HEPA- and charcoal-filtered, with 10-15 changes per hour of 100% filtered fresh air. Room temperature, relative humidity, and airflow were checked at least twice daily.

4.2.5 Necropsy Procedure

A standard necropsy procedure was performed on all test animals as described in Section 5.2.3.

4.3 SALMONELLA TYPHIMURIUM MUTAGENESIS ASSAY

Methods used were those described by Maron and Ames, Mutation Research 113, 175-213, 1983.

4.3.1 Confirmation of Tester Strain Genotypes

The genotypes of tester strains TA100 and TA98 were tested: (a) immediately after receiving the cultures from Dr. Bruce Ames; (b) when a new set of frozen permanents was prepared; (c) with each assay.

The His$^-$ character of the strains was demonstrated by histidine requirement on selective (his$^+$ and his$^-$) growth plates. (The strains also required biotin because of the *uvr*B deletion which extends through the biotin gene). A positive result for histidine requirement was heavy growth on the his$^+$ plates with growth of spontaneous revertants only on the his$^-$ plates. Spontaneous revertants were counted to assure that numbers for all strains were within historical controls.

Presence of the *rfa* (deep rough) mutation in the strains was confirmed by the ability of crystal violet to penetrate the cell wall. Ten microliters of crystal violet was placed in the center of a his$^+$ plate which had been spread with the tester strain. A positive result was a zone of inhibition around the crystal violet drop.

The presence of the *uvr*B deletion was confirmed by plating the strains on his$^+$ plates and irradiating half of the plates for 8 seconds. Strains grew only on the unirradiated sides of the plates.

Presence of the R factor plasmid in the tester strains was determined by testing for ampicillin resistance. Strains were streaked on his$^+$ plates containing ampicillin - one resistant strain and one sensitive strain (as a control) on each plate. TA100 and TA98 both grew on the ampicillin plates (while the sensitive strain did not), indicating the presence of the plasmids in both strains.

4.3.2 Storage of Tester Strains

Stock cultures of the strains were prepared by adding 4.4 ml DMSO to a 50 ml overnight culture, pipetting 1 ml aliquots into half-dram vials, freezing rapidly in dry ice/ acetone or liquid nitrogen, and storing at -80°C.

4.3.3 Growth of Overnight Cultures

An overnight culture was prepared by thawing one frozen stock culture (Section 4.3.2), innoculating a ten microliter aliquot into 50 ml of Oxoid nutrient broth #2, and growing for 10 hours at 37°C and 100 RPM.

4.3.4 Media

Base layer agar plates were prepared in batches using 22.5 g Difco Bacto agar, 30.0 g glucose, 1.5 liter distilled deionized water, and 30 mls of sterile salt solution (Vogel-Bonner Medium E). The salt solution contained 10 g $MgSO_4 \cdot 7H_2O$,100 g citric acid monohydrate, 500 g K_2HPO_4 (anhydrous), and 175 g $Na(NH_4)HPO_4 \cdot 4H_2O$ per liter of distilled deionized water. Each prepared plate contained 20 ml of medium.

His^- agar overlays contained 5 g NaCl, 6 g Bacto-Agar purified, and 100 ml His/Bio Solution per liter of distilled deionized water. His^+ agar overlays contained 250 ul 1N NaOH and 48 mg L-histidine • HCl monohydrate in addition to the above ingredients. His/Bio Solution contained 61.8 mg D-biotin, 48.0 mg L-histidine•HCl monohydrate, and 500 ml distilled deionized water.

4.3.5 S9 and S9 Mix

The S9 fraction was prepared from male Sprague-Dawley rats injected five days before sacrifice with a single intraperitoneal injection of Aroclor 1254, at a dosage of 500 mg/kg. A sterility check of the S9 preparation was performed by inoculating 50 ml nutrient broth with 100 ul S9 fraction, incubating at 37°C for 48 hrs, and checking for bacterial growth. The metabolic activation potential of each S9 batch was also checked. A QC assay was done, similar to the standard mutagenicity assay. Two concentrations of S9 (10% and 2%) were checked using two known mutagens [benzo(a)pyrene and 2-anthramine] at 4 dose levels (0.1, 0.5, 1.0 and 2.5 ug/plate). The metabolic activation was determined by comparison of the results of this assay with historical control values for S9 activation.

For these experiments, S9 mix was prepared at a concentration of 0.05 ml or 0.10 ml of S9 per ml of mix (5% or 10% respectively). Each 50 ml preparation of S9 mix contained 25.0 ml of 0.2M Sodium Phosphate Buffer pH 7.4, 1.0 ml of a 1.65M KCl/0.4M $MgCl_2$ Salt Solution, 1.25 ml of 0.2M Glucose-6-Phosphate, 1.0 ml of 0.2M NADP, 2.5 ml or 5.0 ml of S9 fraction, and 19.25 ml or 16.75 ml of distilled deionized water.

4.3.6 Test Method

The test method employed was the standard plate incorporation test in which bacteria, test chemical, and buffer or S9 mix were plated on selective media in histidine-negative overlays. Poured plates were then incubated for 48 hours at 37°C in a variable temperature incubator. The incubators were on timers set for 48 hours at which time the incubator automatically dropped to 10°C. Positive controls, negative controls, sterility checks, and strain checks were included with each assay. Two modifications of the Maron and Ames method were employed: (1) pre-mixed solutions of bacteria-S9 mix and bacteria-buffer were prepared at the time of experiment initiation and added to the his^- overlays; (2) the amount of test sample volume added to test plates varied, in different experiments, from 100 to 400 ul.

4.3.7 Counting Procedure

Assay plates were counted electronically with an Artek 800 ^{R} automatic cell counter. The Artek counter was calibrated by comparing counts on a plastic control disk to hand counted plates with a known number of different sized colonies. The calibration was done before, during and after the assay was counted. Machine plate counts were also periodically verified by hand to assure that no significant drifting of the machine calibration had occurred.

4.4 SENCAR MOUSE SKIN BIOASSAY: INITIATION/PROMOTION

4.4.1 Source of Animals

Female Sencar mice (strain CR:ORL SENCAR) four to six weeks of age were purchased from the animal production facilities of the NCI-Frederick Cancer Research Facility (Frederick, Maryland).

4.4.2 Receipt of Animals

Upon receipt, the animals were weighed and divided into groups by body weight in increments of 1 gram.

4.4.3 Quarantine Procedures

Animals were placed in isolation for approximately two weeks prior to test initiation. During that time, they exhibited no mortality nor clinical signs of disease. The FCRF facility was reported to be free of all murine viruses and blood serum samples tested at MAI showed no detectable antibodies against Reo 3, K, Polyoma, MVM, Mouse Adenovirus, LCM, PVM, Sendai, Mouse Hepatitis, Ectromelia, or GDVII viruses. After consideration of all observational data, supplier QC data, and serology data by the Study Director and the Director of Veterinary Medicine, the animals were released from quarantine and prepared for test use.

4.4.4 Animal Care

For each study, during the second week of quarantine, the individual animals from each successive body weight group were randomly distributed to 28 cages of 10 animals each and the 28 cages then randomly assigned to one of nine test groups. Each experimental animal was assigned a unique animal number and identified by the use of numbered ear tags.

During the 14 day isolation period as well as the 30 week duration of the study all animals were housed 10 per cage in polycarbonate cages (Lab Products, Garfield, N.J.) offering 15 sq. in. of floor space per animal. All cages were outfitted with stainless steel perforated lids and stainless steel hanging feeders for dispensing pelleted feed. Each cage was identified with cage cards color-coded for each experimental group according to initiator treatment. Data relevant to the occupants of the cages were listed on each card including: study number, strain, sex, treatment, birth date, date of receipt, individual animal numbers, death or sacrifice dates and other experimental data. Each cage card contained color coded tapes designating promotion with 12-0-tetradecanoylphorbol-13-acetate (TPA) or acetone vehicle. The bedding material used in each cage was heat-treated hardwood chips (Northeastern Products Corp., Warrensburg, N.Y.) or 1/8" corn cob bedding (Anderson Cob Mills).

Purina Laboratory Chow Formula #5001 (Ralston Purina Company, St. Louis, Mo.) was offered _ad libitum_ throughout the entire isolation and experimental period. Drinking water was offered _ad libitum_ from glass water bottles and consisted of deionized water generated by a Millipore Milli-RO-60 system (Bedford, Mass.). All animals were offered deionized water from receipt until terminal sacrifice. Both the sources of feed and water have been used routinely in other carcinogenicity studies. The feed and water were not analyzed for specific contaminants.

All animals were changed to sanitized and freshly bedded cages twice per week at which time their water bottles were sanitized and refilled with deionized drinking water. All animals were checked twice a day during the normal work week and daily on weekends and holidays for morbidity, moribundity, and the adequacy of food and water.

All animals were held in animal rooms designed to control for temperatures of 75°F ± 5°F, relative humidities of 50% ± 20%, and light:dark cycles of 12 hours:12 hours (lights on from 700 to 1900 hrs). Temperatures and relative humidities were recorded three times a day on normal work days and once a day on weekends and holidays. Some intermittent excesses of temperature and humidity were noted but were of short duration and not considered extreme enough to affect the quality or integrity of the study. All experimental groups were housed together and experienced identical environmental conditions.

4.4.5 _Initiator Dose Preparation and Initiator Treatment_

All initiation was carried out by intragastric intubation starting when the animals were 6-8 weeks of age. Animals dosed with one of the three test articles or the test article vehicle received 0.5 ml of the appropriate solution as supplied by the sponsor three times per week (M,W,F) for two weeks. A separate 1 1/2" X 20 gauge ball-tipped stainless steel feeding tube attached to a repeating Cornwall syringe was used for each group receiving a test article or the test article vehicle. The delivery apparatus was thoroughly flushed with distilled water between each dosing day. Test article and vehicle solution stocks as well as pre-aliquoted quantities (35 ml and 45 ml, respectively) for each of the six dosing days were stored in amber glass vessels at 4°C ± 3°C prior to use, allowed to reach room temperature prior to administration, and residual quantities returned to 4°C storage. Residual amounts of the aliquots were not reused for dosing.

The positive control group, which received a single dose of urethane on the final day of test article initiation (i.e. 6th dosing day), was dosed intragastrically with a 1 1/2" X 20 gauge feeding tube attached to a calibrated disposable tuberculin syringe using a unit employed for that group only. Just prior to dosing the positive controls, a solution of 25 mg urethane/ml in deionized water was prepared by dissolving 500 mg urethane (Lot 7P07, MCB Manufacturing Company, Cherry Hill, N.J.) in 20 ml of water generated by a Millipore Milli-RO-60 system. Each animal dosed with urethane received 0.02 ml/gm b.w. The final dosage per animal resulting from this procedure was 500 mg/kg b.w. Stocks of urethane were stored at room temperature in amber glass containers. Urethane dose preparations were not analyzed for urethane content and homogeneity, and urethane stocks were not analyzed for purity and urethane content.

4.4.6 Promoter Dose Preparation and Promoter Treatment

Three days prior to the first application of promoter or promoter vehicle, the hair from a standardized area of the backskin was removed using an electric animal clipper (#40 blade). This operation was continued weekly during the remaining 26 week course of experimentation in order to facilitate the scoring and mapping of gross skin tumors and the unobstructed application of TPA or acetone. During the 20 week promotion period all shavings were conducted on intervening days between promotions.

Beginning two weeks after the last dose of the test and control articles, all animals were treated topically with 1 ug TPA in 0.2 ml acetone or 0.2 ml acetone only applied to the shaved backskins three times per week (M,W,F) for 20 weeks. Thereafter all promoter treatments were discontinued for the six week period until test termination.

Stocks of TPA (Chemicals for Cancer Research, Eden Prairie, Minn.) were stored in the dark at -20°C ± 10°C within air-tight sealed glass ampules over desiccant. Dosing solutions of TPA were prepared by dissolving 10 mg TPA in 2000 ml of acetone (Reagent Grade, Baker Chemical Company) and stored in foil-wrapped glass reagent bottles fitted with glass stoppers. Dosing solutions of TPA were stored at -20°C ± 10°C between administrations. Each topical promotion with TPA was carried out by dispensing 0.2 ml of the dose-preparation (1 ug TPA) from an automatic repeating pipettor. Vehicle controls were treated identically except for the use of 0.2 ml acetone only. Disposable pipettor tips were changed between groups receiving TPA or acetone. Stocks of TPA were not analyzed for purity, and dose preparations of TPA were not analyzed for TPA content and homogeneity.

4.4.7. Necropsy Procedure

A standard necropsy was performed on all animals as described in Section 5.4.2.

Ten percent neutral buffered formalin was prepared by mixing 2000 ml of formalin (37-40% formaldehyde) with 18000 ml tap water, and then completely dissolving 80 gms Sodium Phosphate (Monobasic) and 130 gms Sodium Phosphate (Dibasic) in each 20000 mls of 10% formalin prepared.

4.4.8 Preparation of Paraffin Blocks and Stained Slides

Standard histopathology procedures were employed by Pathology Associates, Inc. (PAI) for the preparation of paraffin embedded blocks of gross lesions and for subsequent production of 5 u Hematoxylin and Eosin (H and E) stained slides.

4.5 RAT LIVER FOCUS ASSAY

4.5.1 Source of Animals

Male Sprague-Dawley rats (Strain CR1:COBSCD(SD)Br) were purchased from Charles River Breeding Laboratories, Wilmington, Mass.

4.5.2 Receipt of Animals

Upon receipt, the animals for each study were weighed and randomly distributed to cages. The cages were then randomly assigned to test groups. One group of randomized animals was designated as extras. This group was subjected to partial hepatectomies along with the test groups and subsequently used as replacements for: (1) experimental animals which died from acute toxicity following the intragastric dosing of the test articles or diethylnitrosamine (DEN) (2) animals in which the extent of regurgitation of the intragastric dose resulted in uncertainties as to the delivered dose, or (3) animals with surgical complications (hernias at the suture line which could not be resutured).

4.5.3 Quarantine Procedures

Animals were placed in isolation for approximately two weeks prior to test initiation. During that time, they exhibited no mortality nor clinical signs of disease. Blood serum samples from randomly selected rats were checked for antibody titers to the following murine viruses: Reo-3, GDVII, KRV, H-1, Mouse Adenovirus, LCM, PVM, Sendai, and RCV/SDA. All results were negative. After consideration of all observational data, supplier QC data, and serology data by the Study Director and the Director of Veterinary Medicine, the animals were released from quarantine and prepared for test use.

4.5.4. Animal Care

Each experimental animal as well as the replacements utilized in the studies were assigned unique animal numbers and identified by the use of numbered ear tags at the time the test was started.

During the 14 day isolation period as well as the 10 week duration of the study, all animals were housed 3 to 4 per cage in polycarbonate cages (Lab Products, Garfield, N.J.) offering 40 sq. in. of floor space per animal. All cages were outfitted with stainless steel wire bar lids with intergrated feeders for dispensing pelleted feed. Each cage was identified with cage cards color-coded for each experimental group. Data relevant to the occupants of the cages were listed on each card including; study number, strain, sex, treatment and treatment dates, birth date, date of receipt, individual animal numbers, death or sacrifice dates and other experimental data. All cage cards also contained color coded tapes which were matched to color codes used to identify carboys of deionized water or water containing phenobarbital (PB). The inclusive dates of treatment for each drinking water solution were specified on the tape. The bedding material used in each cage was heat-treated hardwood chips (Northeastern Products Corp., Warrensburg, N.Y.).

Purina Laboratory Chow Formula #5001 (Ralston Purina Company, St. Louis, Mo.) was offered _ad libitum_ throughout the entire isolation and experimental period. Drinking water was offered _ad libitum_ from polycarbonate water bottles and consisted of deionized water generated by a Millipore Mili-RO-60 system (Bedford, Mass.) or deionized water containing 0.05% PB. All animals were offered deionized water from receipt until 7-9 days following partial hepatectomy. From that time until 8 weeks later they received deionized water containing 0.05% PB, at which time they were switched back to deionized water for 1 week until the animals were sacrificed and the test terminated. Both the source of feed and water have been used routinely in other carcinogenicity studies. The feed and water for these studies were not analyzed for specific contaminants.

All animals were changed to sanitized and freshly bedded cages twice per week at which time their water bottles were sanitized and refilled with deionized drinking water or PB-dosed deionized water. All animals were checked twice daily during the normal work week and once daily on weekends and holidays for morbidity, moribundity, and the adequacy of food and water.

All animals were held in animal rooms designed to control for temperatures of 75°F ± 5°F, relative humidities of 50% ± 20%, and light:dark cycles of 12 hours:12 hours (lights on from 700 to 1900 hrs). Temperatures and humidities were recorded three times a day on normal work days and once a day on weekends and holidays. Some intermittent excesses of temperature and humidity were noted but were of short duration and not considered extreme enough to effect the quality or integrity of the study. All experimental groups were housed together and experienced identical environmental conditions.

4.5.5 Partial Hepatectomies

For each study, all animals received partial hepatectomies. Each animal was anesthetized with ethyl ether (Mallinckrodt Chemicals) in a bell jar contained under a charcoal filtered fume hood, subsequently maintained under anesthesia through the use of an ether cone, and the surgical field shaved with an electric animal hair clipper and swabbed with alcohol. A 2 cm longitudinal midline incision was made through the abdominal skin and peritoneal wall just below the sternum. Sterile saline-soaked 2" x 2" gauge was used to drape the field and the liver extruded through the incision by exerting pressure with thumbs placed below the incision and fingers placed on the dorsal peritoneal wall. An alcohol-sterilized 1-0 silk loop was placed around the base of the median and left lateral lobes. The mesentery at the base of those lobes was carefully snipped with sterile scissors without damaging the vasculature entering the lobes, the silk loop was knotted and the lobes ligated by securely tightening the knotted silk. The ligated lobes were then cut off with sterile scissors and excess thread from the ligature cut off distal enough from the knot to prevent loss of the ligature. The peritoneal wall was then closed by suturing with 3-0 surgical gut and the skin sutured with 3-0 surgical silk. The external skin around the incision site was swabbed with alcohol, the anesthesia cone removed, and the animal returned to its appropriate cage to recover.

The use of surgical silk (rather than wound clips) and the use of alcohol (rather than weak iodine solution) to disinfect the external skin were minor deviations from the original EPA Statement of Work. In addition, in both studies (Nine Sample Series, MAI Assay Report 1840.02 and Five Sample Series, MAI Assay Report 1840.05), the range of body weights at the time of partial hepatectomy exceeded that originally requested in the EPA Statement of Work (175-225 g). This occurred due to the fact that although the animals gained weight at rates greater than expected from the breeders growth curves for the strain, a minimum isolation period was still maintained to verify the health of the animals prior to test initiation. It was not felt that this deviation affected the quality or integrity of the study since all treatment groups (both test and control) were within the heavier weight range. In the Nine Sample Study only (MAI Assay Report 1840.02), due to the large number (123) of partial hepatectomies performed and the fact that timed intragastric treatments were required precisely 24 hours after hepatectomy, surgery was performed on one half of each experimental group and one half of the extra animals on each of two days, April 9 and April 11, 1984.

4.5.6 Preparation and Storage of Positive Control

Prior to dosing the animals in the positive control group, solutions of 3.3 mg DEN/ml were prepared in sterile normal saline (0.85% saline). DEN (Lot # 100F-0574) was purchased from Sigma Chemical Company (St. Louis, MO.), and sterile normal saline purchased from M.S. Bioproducts (Walkersville, Md.). Each animal dosed with DEN received 0.15 ml per 10 gms body weight to yield a dose of 50 mg DEN/kg body weight. Stocks of

DEN were stored at 4°C in light tight vessels and dosing solutions allowed to come to room temperature prior to administration. Dose preparations of DEN were not analyzed for DEN content and homogeneity, and DEN stocks were not analyzed for purity and DEN content.

4.5.7 Initiator Treatment Method

All animals were treated with initiator by intragastric intubation precisely 24 hrs after partial hepatectomies. Animals dosed with test article or test article vehicle (2% Emulphor) received 3.0 ml of the appropriate solution, using a separate 3" x 18 gauge ball-tipped stainless steel feeding tube attached to a calibrated 3 ml disposable syringe for each test group. Solutions were stored at 4°C ± 3°C prior to use, allowed to reach room temperature prior to administration, and returned to 4°C storage after dosing was completed. The positive control group which received DEN was dosed with the same type dosing apparatus described above employing a unit used for that group only. Residual dosing solutions of DEN were discarded.

4.5.8 Promoter Dose Preparation and Promoter Treatment

Stocks of anhydrous PB (Lot #23F-0704) were ordered from Sigma Chemical Company (St. Louis, Mo.) and stored at room temperature. Phenobarbitol-dosed water was prepared by combining 10 gm of PB with 20,000 ml deionized water obtained from a Millipore Milli-RO-60 system (Millipore Corporation, Bedford, Mass) in carboys, and disolving the PB by placement of the carboy on a mechanical platform shaker. Carboys were color coded for deionized and PB-dosed deionized water as were the animal cages receiving those solutions. Dosed water preparations were stored in the animal rooms at room temperature and were consumed within a maximum of 7 days after mixing.

Seven to nine days following partial hepatectomy, all animals were offered a solution of 0.05% (W/V) PB in deionized water as their drinking water for a period of 8 weeks. The administration of deionized water only was resumed after that time and continued for a period of one week at which time all surviving animals were sacrificed and evaluated.

Although the Statement of Work called for promotion to start 7 days after surgery, in the Nine Sample Study (MAI Assay Report 1840.02), it was started on the same day for both cohorts of animals with surgery dates separated by 48 hours and resulted in promotion starting 7-9 days after surgery. The total duration of promotion was as required (56 days) with sacrifice 1 week after removal of the promoter. This was done so that confusion in starting different animals in the same cage on promoter on different days was not encountered. The fact that animals in the same cage had different surgery dates resulted from the splitting of groups in half for surgery on two days and the fact that when replacements were used for early losses, due to toxicity or regurgitation of the initiators, those used as replacements did not necessarily have the same surgery date as their cagemates. Since initiation is a persistent event, the two day range over which promotion was started was not deemed as important as avoiding confusion in the maintenance of proper animal identification and experimental records as long as the timing of initiator treatment was always 24 hours after surgery, the length of promotion was equivalent for all animals, and the time of terminal sacrifice measured from the start of promotion was equivalent for all animals. The accuracy of these three later important factors was verified and supported by the raw data records. The pattern of GGT+ focus formation reported later for the positive controls seemed to verify the prudence of the method.

In the Five Sample Study (MAI Assay Report 1840.05), promotion of all the animals began 7 days following the partial hepatectomies as specified in the EPA Statement of Work.

4.5.9 Procurement and Preservation of Tissues

One week after completion of promoter treatment, liver samples were collected from all surviving animals. Necropsies and the preservation of tissues by fixation or freezing were not routinely carried out for early deaths or animals sacrificed before the end of the study. Liver samples from animals reaching the terminal sacrifice were processed for the preparation of frozen sections and low melting point (43°C) paraffin embedded blocks.

Rats were sacrificed by carbon dioxide inhalation (rather than decapitation, EPA Statement of Work). The right lateral lobe from each animal was quickly excised, rinsed in 0.85% sodium chloride, and trimmed to yield a series of four 2 mm thick sections and a series of four 1 mm thick sections. All sections were additionally trimmed so that parenchymal surfaces measured approximately 1 cm^2.

The four 2 mm thick sections from each animal were individually placed in 25 x 20 x 5 mm cryomolds containing OCT cryopreserving media, rapidly frozen on dry ice, and the blocks stored at -70°C ± 10°C until sectioned and stained. The temperature of the ultrafreezer used for storage was not -80°C as required by the EPA Statement of Work but maintained enzyme activity as intended. Each cryomold was labelled with the appropriate date, study number, and animal number. Each section of liver was placed in the mold and oriented such that the sectioned faces of each block were not serially related adjacent surfaces.

The series of four 1 mm thick sections from each animal were placed in jars containing 95% ethanol - 1% acetic acid, which had been chilled to 4°C, and placed on wet ice for 30 minutes. The individual sample jars for each animal were identified by date, study number and animal number. Following the 30 minute period of fixation in ethanol-acetic acid, the tissues were placed in absolute ethanol at 4°C ± 3°C until processed for the production of low melting point paraffin embedded blocks.

4.5.10 Histology Procedures for Paraffin Block Production

All ethanol-fixed liver samples were passed into 2 changes of xylene (2 hours and 1 hour, respectively), infiltrated with low melting point paraffin for no longer than 24 hours, and individually embedded in blocks of low melting point paraffin. All resulting blocks were identified by the appropriate group and animal numbers. Such blocks were stored at 4°C ± 3°C until supplied to the sponsor at test termination.

4.5.11 Frozen Microtomy Procedure

Frozen tissue blocks of liver with study number and animal number were delivered to Pathology Associates, Inc. (PAI) by Microbiological Associates, Inc. (MAI). Upon receipt the blocks were inventoried and stored in a -80°C freezer until they were sectioned into 6 micro-meter sections, using a cryostat, at -25°C. Instead of using albumin coated slides as described in the EPA Statement of Work, prelabelled gelatin coated "subbed" slides were used to "pick up" the sections.

4.5.12 Gamma Glutamyl Transpeptidase (GGTase) Staining Procedure

The staining procedure used by PAI was as described in the EPA Statement of Work except that: 1) the Fast Blue BB salt was mixed with 20 ml of incubation solution rather than 19 ml, 2) the incubation solution was more concentrated, and 3) the glycerin mounting medium used phenol instead of merthiolate (ethyl-mercurithiosaliacylate). These modified procedures (described in this section and section 4.5.11 above) were used because they have been successfully used since the inception of the EPA rat liver focus assay approximately five years ago.

4.5.13 Examination of Stained Slides

The sections (4 per animal) were examined using a light microscope with an ocular grid, using the point system as described by Weible (attached to MAI Assay Reports). Briefly, the grid was placed over the liver section and the number of points covering the section were counted as were the number of points covering the areas of zonal induction (ZI) of GGTase activity. The radius of each GGTase positive focus with greater than nine nuclei was measured and the number of GGTase positive foci with greater than nine nuclei were counted. This information was recorded on a Slide Evaluation Sheet.

4.5.14 Mathematical and Statistical Methods

For each animal, the area of liver examined and the area of ZI was determined by multiplying the number of points counted by 0.0025 cm^2 (the area represented by one point as determined with a micrometer). Only animals in which a minimum of two cm^2 of liver was evaluated were used in later compiling the group data. The area of each focus was determined by the formula $\pi(r^2)$ = Area. The total GGTase positive foci area per animal was equal to the sum of all the individual foci areas. The number of foci per cm^2 was determined by dividing the number of foci counted per animal by the area of liver evaluated per animal. The number of foci per cm^3 was determined for each animal by the formula $Nv = 1/r1 + 1/r2 + 1/r3 \ldots 1/rn / \pi(A)$. The percentage of ZI was determined by dividing the area of liver exhibiting ZI per animal by the area of liver evaluated per animal. The percentage of GGTase positive foci was determined by dividing the total GGTase positive foci area per animal by the area of liver evaluated per animal. The standard error of the mean was determined by dividing $\sqrt{n}$ by the standard deviation.

A two-tailed *t*-test for comparison of two means was used to analyze the group data (reference included in MAI Assay Reports).

4.6 LUNG ADENOMA ASSAY IN STRAIN A MICE

4.6.1 Source of Animals

Six-week old virus and parasite-free Strain A/J mice (male and female) weighing 18-22 grams were purchased and received from Jackson Laboratories, Bar Harbor, Maine.

4.6.2 Receipt of Animals

Upon receipt, animals were randomly assigned to test groups, caged, and permanently identified with ear punch numbers.

4.6.3 Quarantine Procedures

During a subsequent two-week quarantine period, four randomly selected mice (2 males and 2 females) were checked for ectoparasites and antibody titers against eleven known murine viruses. All results were negative. Animals were observed daily for signs of illness or death. On the last day of the quarantine period, the Staff Veterinarian reviewed the data from the animal supplier, the serology data, and the results of the daily health checks. All animals judged healthy and suitable as test animals were released from quarantine.

4.6.4 Animal Care

Males or females, four each per cage, were housed in plastic or stainless steel cages (approx. 10 1/2" x 8 1/2") with stainless steel tops. Water was supplied by either water bottles or an automatic watering system. Cages and bedding were changed weekly. Each cage was identified with color-coded cage cards indicating the experimental group and bearing all pertinent data as to dates of treatment, sex, and unique consecutive numbers of the caged animals. Food and water were offered *ad libitum*. Water supplied was deionized water. Purina Certified Laboratory Chow #5002 was used as the basal diet, supplied in stainless steel hoppers placed inside cages. Food and water bottles were provided fresh every week. Temperature in the animal room was held at 20°C ± 5°C and humidity at 50% ± 10%. Light was controlled automatically by a timer yielding 12 hr cycles of light-darkness. Air entering the room was HEPA- and charcoal-filtered, with 10-15 changes per hour of 100% filtered fresh air. Room temperature, relative humidity, and airflow were checked at least twice daily.

4.6.5 Preparation and Storage of Positive Control

Urethane stocks were obtained from the EPA and stored at 4°C in light-tight vessels. Just prior to use, the stock solution was diluted with sterile distilled water to yield 10 mg urethane in 0.5 ml.

4.6.6 Sample Treatment Method

All animals were dosed by gavage with 0.25 ml of sample or solvent control 3X per week for 8 weeks or with 0.50 ml of positive control (10 mg urethane) in a single dose using a 1.5" x 20 gauge curved ball-tipped stainless steel feeding tube attached to a 1 ml disposable syringe. A separate tube and syringe were used for each treatment group.

4.6.7 Necropsy Procedure

A standard necropsy was performed on all test animals as described in Section 5.6.2.

4.6.8 Preparation of Stained Slides for Adenoma Counts

Standard histopathology procedures were employed by PAI for the preparation of stained lung section slides.

SECTION 5

EXPERIMENTAL PROCEDURES

5.1 SAMPLE PREPARATION AND STORAGE

5.1.1 Eight Sample Series

Water samples 1-6L-P74 through 8B-2G-B-1+B-2-P60 (Table 4-1) were obtained from Robert Miller, USEPA, on 19 January 1984. Samples 1-6L-P74 through 5-6L-P82 were diluted with sterile distilled water to yield sample concentrations of 1X and 1/4X. Samples 6A-2G-A-1+A-2-P70 through 8B-2G-B-1+B-2-P60 were tested as provided by EPA (6A, 7A, 8A = 1X concentration; 6B, 7B, 8B = 1/10X concentration). Aseptic techniques were used during all sample handling. During the studies, all samples and dilutions were stored at 4°C.

5.1.2 Nine Sample Series

A series of nine organic acetone samples were obtained from Robert Miller, USEPA, on 19 January 1984 (Table 4-1). Each 5 liter sample was contained in amber gallon jugs with approximately 2.5 liters per container. Upon receipt, 150 mls of sample was removed from each jug and stored at -20°C for a later concentration (3-26-84). The remaining sample was stored at -20°C for 24 hrs, equilibrated to room temperature for 24 hrs, and then concentrated on 1-21-84 by removal of the acetone solvent.

1-21-84 Concentration--

Concentration of five liter volumes was achieved by vacuum distillation at 45°C using a series of cold traps for the volatilized acetone. Peak removal rates were approximately 300-400 ml/hr. After concentration, sterile deionized water was added to bring the volume to 1/5 that of the starting volume. Sterile 10% Emulphor was then added (1 volume to 4 volumes of sample) and the samples aliquoted for the various assay procedures.

For the Ames *Salmonella typhimurium* mutagenesis assay, four aliquots of 20 ml each per sample were placed in amber vials and labelled as follows: two with test sample number and an A series designation and two with test sample number and a B series designation. The samples were then transported overnight on ice to RTP, N.C. and stored at 4°C until tested. Samples were diluted on the test day with sterile 2% Emulphor to yield the appropriate sample doses. For the Sencar and rat liver focus assays, 225 ml and 50 ml, respectively, of each sample were shipped at ambient temperature to Dr. M. Wenk at MAI. Upon receipt, samples were stored at 4°C. For the Sencar assay, samples were used as supplied without dilution with the exception of sample #6 which was diluted prior to test use with sterile 2% Emulphor to yield a 1/2X sample concentration. For the rat liver focus assay, all samples were used as supplied without further dilution. For the lung adenoma assay, 800 ml of each sample was stored at 4°C and prior to test use, diluted with sterile 2% Emulphor to yield concentrations of 1/2X and 1/4X (sample 6) or 1X and 1/2X (all other samples).

3-26-84 Concentration--

On 3-26-84 aliquots of 50 ml were removed from each of the 9 (150 ml) sample volumes stored at -20°C from 1-19-84. Concentration was achieved by vacuum distillation at 45°C using an EPA concentrator apparatus. After concentration, sterile deionized water was added to bring the volume to 10 ml. Sterile 10% Emulphor was then added (2.5 ml/10 ml concentrate). Samples for Ames testing were transferred to amber vials (with C series designation), placed at 4°C, and transported overnight to RTP, N.C. Samples were stored at 4°C until tested.

Table 5-1 summarizes the concentration results showing the starting and final volumes and the additions to yield a final 4X concentration of the starting material.

5.1.3 Eighteen Sample Series

A series of 18 organic acetone samples were obtained from Dr. Robert Miller, USEPA, on 19 January 1984 (Table 4-1). Each sample was contained in amber vials with approximately 80 ml per container. The samples were stored at -20°C.

On 11 July 1984 all samples were equilibrated to room temperature for 2 to 4 hours and then concentrated by removal of the acetone solvent. Concentration was achieved by heating at 45°C under a nitrogen stream to volatilize the acetone. Peak removal rates were approximately 60 ml/30-45 min. After concentration, sterile water was added to bring the volume to 1/5 that of the starting volume. Sterile 10% Emulphor was then added (1 volume to 4 volumes of sample) to the concentrating flask, the sediment dissolved where feasible, and the samples aliquoted into amber vials for the Ames *Salmonella typhimurium* mutagenesis assay procedure. Twelve samples of 20 ml each and six samples of more than 20 ml were transported overnight to RTP, NC. Samples were stored at 4°C until tested.

Table 5-2 summarizes the concentration results showing the concentrated volume and the additions to yield a final 4X concentration of the starting material where possible. Samples that failed to concentrate to 1/5 the starting volume received 10% Emulphor (1 volume to 4 volumes) to yield a final concentration of 2% Emulphor solvent.

Table 5-1 Concentration of Nine Sample Series

Sample	Initial Sample Concentration	Initial Volume	Concentrated Volume[a] (ml)	Sterile H^2O Added (ml)	Sterile Emulphor Added (ml)	Final Volume in 2% Emulphor (ml)	Final Sample Concentration
1-21-84 Concentration							
1-5L-P96	1000X	5L	860	140	250	1250	4000X
2-5L-P88	1000X	5L	710	290	250	1250	4000X
3-5L-P90	1000X	5L	690	310	250	1250	4000X
4-5L-P92	1000X	5L	670	330	250	1250	4000X
5-5L-P94[b]	1000X	5L	740	260	250	1250	4000X
6-5L-P96	1000X	5L	980	20	250	1250	4000X
7-5L-P98	1000X	5L	810	190	250	1250	4000X
8-5L-P30	1000X	5L	950	50	250	1250	4000X
9-5L-P38	1000X	5L	1210[c]	0	300	1510	3311X
3-26-84 Concentration							
1-5L-P96	1000X	50	4.5	5.5	2.5	12.5	4000X
2-5L-P88	1000X	50	7.5	2.5	2.5	12.5	4000X
3-5L-P90	1000X	50	8.0	2.0	2.5	12.5	4000X
4-5L-P92	1000X	50	7.8	2.2	2.5	12.5	4000X
5-5L-P94	1000X	50	6.7	3.3	2.5	12.5	4000X
6-5L-P96	1000X	50	7.1	2.9	2.5	12.5	4000X
7-5L-P98	1000X	50	8.2	1.8	2.5	12.5	4000X
8-5L-P30	1000X	50	7.5	2.5	2.5	12.5	4000X
9-5L-P38	1000X	50	8.0	2.0	2.5	12.5	4000X

[a] Samples were concentrated by vacuum distillation of the acetone solvent at 45°C.

[b] Bottle #2 of this sample contained white ppt. and remains of a teflon cap liner.

[c] After reaching a volume of 600 ml, bottle #1 could not be concentrated further. Bottle #2 reached a concentration plateau at 660 ml. Additional concentration using a Kuderna-Danish removed 50 ml, but further concentration could not be obtained at the recommended temperatures of 45°C.

Table 5-2 Concentration of Eighteen Sample Series

Sample	Initial Sample Concentration	Volume (ml)	Concentrated Volume[a] (ml)	Sterile H_2O Added (ml)	Sterile Emulphor Added (ml)	Final Volume in 2% Emulphor (ml)	Final Sample Concentration
10-80-P32	1000X	80	10	6	4	20	4000X
11-80-P34	1000X	80	9	7	4	20	4000X
12-80-P36	1000X	80	13	3	4	20	4000X
13-80-P40	1000X	80	11	5	4	20	4000X
14-80-P42	1000X	80	14.5	1.5	4	20	4000X
15-80-P14	1000X	80	10	6	4	20	4000X
16-80-P16	1000X	80	14	2	4	20	4000X
17-80-P18	1000X	80	10	6	4	20	4000X
18-80-P20	1000X	80	14	2	4	20	4000X
19-80-P22	1000X	80	14	2	4	20	4000X
20-80-P24	1000X	80	8	8	4	20	4000X
21-80-P26[b]	1000X	80	16	0	4	20	4000X
22-80-P46[b]	275X	80	25	0	5	30	733X
23-80-P48[b]	275X	80	20	0	5	25	880X
24-80-P50[b]	275X	80	32	0	8	30	733X
25-80-P52[b]	275X	80	21	0	5	26	846X
26-80-P54[b]	275X	80	19	0	5	24	916X
27-80-P56[b]	275X	80	27	0	7	34	647X

[a] Samples were concentrated by volatilizing the acetone solvent at 45° under nitrogen.

[b] After reaching these volumes, further concentration of the samples could not be obtained at the recommended temperature of 45°C.

5.1.4 Five Sample Series

Five drinking water concentrates (4000X) dissolved in 2% Emulphor were obtained from Robert Miller, USEPA, on 24 September 1984. Sample designations and volumes were as follows: sample 1 - 9/84, 1 liter; sample 2 - 9/84, 1 liter; sample 3 - 9/84, 1 liter; sample 4 - 9/84, 1 liter; 5 - 9/84, 1 liter.

Samples were aliquoted for the various assay procedures. For the *in vivo* toxicity assay, the samples were stored at 4°C, and then prior to test use, diluted with sterile 2% Emulphor to yield sample concentrations of 1X and 1/4X. For the Ames assay, one 10 ml aliquot of each of the samples was placed in a sterile amber vial, transported overnight to RTP, NC, and stored at 4°C until tested. Samples were diluted on the test day with sterile 2% Emulphor to yield the appropriate sample concentrations. For the rat liver focus assay, 40 ml of each sample were shipped at ambient temperature to Dr. M. Wenk at MAI. Upon receipt samples were stored at 4°C. Samples were used as supplied without further dilution. For the lung adenoma assay, 50 ml of each sample were stored at 4°C and prior to test use, diluted with sterile 2% Emulphor to yield sample concentrations of 1X and 1/2X.

5.2 *IN VIVO* TOXICITY TEST IN MICE

5.2.1 Test Parameters

For each study, CD-1 mice (approximately 8 weeks of age) were exposed to test samples (or control) for a period of 30 days. Two dose groups, receiving 1X and 1/4X or 1/10X concentrations, were employed per test sample. The control group of mice received sterile deionized water or sterile 2% Emulphor. Each test or control group contained a total of 20 mice - 10 male and 10 female. At the conclusion of the 30 day treatment period, all mice were sacrificed and necropsied.

5.2.2 Mode of Administration

In Study 1 (February/March 1984, 8 samples), the test samples (or control) were administered in the drinking water, with only about a 3 or 4 day supply of water (approximately 35 mls/day/cage) placed in the bottle twice weekly.

In Study 2 (October/December 1984, 5 samples), test samples (or control) were administered by gavage, 0.3 ml per mouse, on each Monday, Wednesday, and Friday of the 30 day treatment period (total of 13 doses).

5.2.3 Experimental Observations

Body Weight and Fluid Consumption--

Body weights of all mice were determined at time 0 and then once weekly throughout the 30-day treatment period. These weights were recorded on an appropriate data sheet (Chart 5-1). For Study 1 (employing

Chart 5-1 Body Weights, Food and Water Consumption

STUDY:________________ SPONSOR:____ PROJECT #:________

TEST ARTICLE:____________ ROUTE:______ DATE:Born ______ Received ________

TEMP I.D. #: ___-___ T. CAGE #:T-_____ PERM. I.D. #M-________ P. CAGE #P-______

PHASE	d__to__	Date____to____	TREATMENT	CAGEMATE'S ID #	CAGE #
QUARANTINE		to	N/A		T-
QUARANTINE		to	N/A	M-	P-
TASK -		to		M-	P-
TASK -		to		M-	P-
TASK -		to		M-	P-
TASK -		to		M-	P-
TASK -		to		M-	P-
TASK -		to		M-	P-

BODY WEIGHT

DATE	d	b.w.	Sign.	
		(g)		

COMMENTS:

FEED/WATER CONSUMPTION

d__to__	In.Wt.	Sig	F.Wt.	Sig	#An	Amt.	Amt/Day
to	(g)		(g)			(g)	(g)
to							
to							
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drinking water as the mode of sample administration)), water consumption was measured twice weekly and recorded (Chart 5-1). Water consumption was not measured during Study 2.

Health Status Checks--

A cage-side observation of each animal for overt signs of illness (Table 5-3) or death was performed twice daily and any abnormal findings recorded on its Health Surveillance Record form (Chart 5-2).

A detailed health status check was performed on each mouse once a week at the time of the weekly weighing. The type, frequency and severity of any toxic signs was recorded (Chart 5-2) in code form (Chart 5-3) along with the initials of the observer and the date of observation.

Necropsy--

At the end of the 30-day treatment period, all mice were weighed, sacrificed (by CO_2 overdose in Study 1; by cervical dislocation in Study 2), and necropsied. Animals found dead or moribund during the treatment period were also necropsied. The liver, lungs, kidneys, spleen, brain, and gonads of each animal were examined, weighed, and preserved in individually labelled specimen bottles containing 10% buffered formalin. All findings were recorded on Necropsy Summary Sheets (Chart 5-4).

5.2.4 Analysis of Data

Using the individual body weights and organ weights, a mean, standard deviation, and standard error were calculated for the male and female mice in each test or control group. Using the per cage water consumption data (Study 1 only), the mean amount of water consumed per animal per week and per day was determined for each test or control group.

5.2.5 Records

All records pertaining to these studies including all original data, correspondence, original and draft copies of reports, and pathology materials were retained and placed in the EHRT Archives at the conclusion of the study. The pathology materials will be released to EPA at their request.

5.3 SALMONELLA TYPHIMURIUM MUTAGENESIS ASSAY

5.3.1 Test Parameters

In each experiment, a series of samples were tested in strains TA98 and TA100 at four sample doses in duplicate with and/or without S9 activation. The amount of TA98 or TA100 added to each test plate was 100 ul of an overnight culture. In each experiment, samples were tested within an eight-fold range with the highest sample dose not exceeding 400 ul per plate. Although the actual amount of test sample added during individual experiments varied, the volume added to each test plate was

Table 5-3 DEFINITION OF PHARMACOLOGICAL TOXIC SIGNS

Organ System	Observation or Examination	Common Signs of Toxicity
CNS and somatomotor	Behavior	Change in attitude to observer unusual vocalization, restlessness, sedation
	Movements	Twitch, tremor, ataxia, catatonia, paralysis, convulsion, forced movements
	Reactivity to various stimuli	Irritability, passivity, anaesthesis, hyperaesthesis
	Cerebral and spinal	Sluggishness, absence of reflexes
	Muscle tone	Rigidity, flaccidity
Autonomic nervous system	Pupil size	Myosis, mydriasis
	Secretion	Salivation, lacrimation
Respiratory	Nostrils	Discharge
	Character and rate of breathing	Bradypnoea, dyspnoea, Cheyne-Stokes respirations, Kussmaul-breathing
Cardiovascular	Palpation of cardiac region	Thrill, bradycardia, arrhythmia, stronger or weaker beat
Gastrointestinal	Events	Diarrhea, constipation,
	Abdominal shape	Flatulence, contraction
	Feces consistency and color	Unformed, black or clay colored
	Perianal region	Soiled
Skin and fur	Color, turgor integrity	Reddening, flaccid skinfold, eruptions, piloerection
Mucous membranes	Conjunctiva, mouth	Discharge, congestion, hemorrhage, cyanosis, jaundice
Eye	Eyeball	Exophthalmus, nystagmus
	Transparency	Opacities
Others	Rectal or paw skin	Subnormal, increased temperature
	General Condition	Abnormal posture, emaciation

Chart 5-2 Health Surveillance Records

TEMP. ID #:____-________ T. CAGE #:T-______ PERM. ID #:_M______________ P. CAGE #:P-________

d__ __	DATES	CODE DESCRIPTION	SIGN	CORRECTIVE ACTION	SIGN

Chart 5-3 Health Surveillance Code

CODE	
N & H	Normal and Health
Miss	Missing
Preg	Pregnant
V-Care	Needs Veterinary Care
Dead	Dead
Cann	Cannibalized
Acci	Accidental Death
Mori	Moribund
Thin	Thin
Trem	Tremors
Obes	Obese
A-Pos	Abnormal posture
Limp	Limping
Twit	Twitching
U-Res	Unresponsive
Para	Paralyzed
Hyp-	Hyperactive
Circ	Circling
Conv	Convulsions
Anor	Anorexia
Leth	Lethargic
Pros	Prostrate
Atax	Ataxic
Adip	Adipsia
P-dip	Polydipsia
Cou-	Coughing
Sne-	Sneezing
Whe-	Wheezing
P-phe	Polyphea
Disp	Dispnea
E-tax	Epistaxis
Rhin	Rhinorrhea
Sore	Sores
S-Wou	Skin Wounds
Rash	Rashes
R-HC	Rough Hair Coat
S-HC	Strained Hair Coat
S-bur	Shane Burns
B-Stn	Blood Stains
A-pic	Alopicia
Cyan	Cyanotic
P-erc	Piloerection
Desq	Desquantation
Tiss	Tissuring
Edem	Edema
Eryt	Erytheme
Esch	Eschar
Mydr	Mydriasis
Mios	Miosis
Squi	Squinted
Opaq	Opaquae
Lacr	Lacrimation
C-dac	Chromodacryorrhea
E-pht	Exophthalmus
Emes	Emesis
Sali	Salivation
Diar	Diarrhea
S-AA	Soiled Anal Area
Anur	Anuria
P-uri	Polyuria
S-fec	Soft feces
C-sto	Colored stool

Chart 5-4 Necropsy Summary Sheet

STUDY:______________ TEST ARTICLE____________ ROUTE________ EXP. ANIMALS_______ SPONSOR____

	ANIMAL #	TREAT-MENT	WEIGHT								OTHER	
			BODY	LIVER	BRAIN	SPLEEN	LUNGS	KIDNEYS	TESTES	OVARIES		
			(g)	(g)	(g)	(g)	(g)	(g)	(g)	(g)	(g)	(g)
MALES												
FEMALES												
	GROUP MEAN											
	STANDARD DEVIATION											
	STANDARD ERROR											

held constant in each experiment by appropriate dilution of the samples with 2% Emulphor. For test plates not receiving S9 Mix (500 or 400 ul), 0.1 M PO_4 buffer was substituted to keep the added volumes constant.

Negative solvent controls were run with each experiment. These controls consisted of 100 or 200 ul 2% Emulphor along with a 100 ul bacterial aliquot and buffer or S9 mix, in appropriate amounts.

Positive controls were run with each experiment. For tester strain TA98, controls were 2-nitrofluorene 1.0 ug and 5.0 ug (without S9 activation) and 2-anthramine 1.0 ug and 2.0 ug (with S9 activation). For tester strain TA100, controls were sodium azide 1.0 ug and 2.0 ug (without S9 activation) and 2-anthramine 1.0 ug and 2.0 ug (with S9 activation).

Sterility checks of the samples, the solvent, the buffer, and the S9 mix were run with each experiment.

In each experiment, the bacterial strains were checked for genetic markers-*rfa* mutation, *uvr*B deletion, and presence of the R factor plasmid.

5.3.2 Experimental Observations

Revertant Counts--

Test plates were incubated in the dark at 37°C for 48 hours and revertants then counted using an Artek 880 automatic colony counter. All findings were recorded on reporting forms supplied by the EPA Project Officer (Chart 5-5).

Toxicity Checks--

The background lawn of the test plates was examined with a stereomicroscope to evaluate toxic effects.

5.3.3 Quality Control Criteria

Values for both the direct-acting and indirect-acting positive control compounds must be at least two-fold greater than the spontaneous background rates for all tester strains. Spontaneous background rates (solvent controls) for the *Salmonella* strains must be within the 95% confidence intervals of historical spontaneous background rates for the assay to be acceptable. Sterility and strain genetic marker checks (i.e., histidine dependence, crystal violet and UV sensitivity, and ampicillin) must be acceptable on each assay date.

5.3.4 Analysis of Data

In all experiments, a positive mutagenic response was indicated by a 2-fold increase at two dose levels or a 2-fold increase at one dose level with a dose-related increase at lower concentrations. No statistical analysis was performed.

Chart 5-5 Ames Test Reporting Form

Experiment No.: ____________
Assay Type: ____________
Sample Name: ____________
Sample I.D.: ____________
Solvent: ____________
Test Initiation Date: ____________
Test Completion Date: ____________
S-9 Type and Lot No.: ____________

Positive Controls	Strain	-S9 Compound	-S9 Amount/Plate	+S9 Compound	+S9 Amount/plate

R E V E R T A N T S   P E R   P L A T E / S T R A I N

Test														
			1	2	3	1	2	3	1	2	3	1	2	3
-S9														
Solvent Control														
Positive Control														
Test Compound														
Dose Level	**Units**													
.														
.														
.														
.														
.														
.														
.														
.														
+S9														
Solvent Control														
Positive Control														
Test Compound														
Dose Level	**Units**													
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5.3.5 Records

All records pertaining to these studies were retained and placed in the RTI Archives at the conclusion of the study.

5.4 SENCAR MOUSE SKIN BIOASSAY: INITIATION/PROMOTION

5.4.1 Test Parameters

Due to the limited ability of the designated animal breeders to supply animals, the testing of the nine sample series (Section 5.1.2) in the Sencar assay was performed as three separate and independent studies. For each study, six-to eight-week-old female Sencar mice were dosed three times a week (M,W,F) for two weeks intragastrically with either 0.5 ml of the test article or 0.5 ml of the test article vehicle. Sample concentrations were 1X (undiluted) for all samples except sample 6 which was tested at 1/2X concentration. Positive controls received a single intragastric administration of urethane in deionized water (500 mg/kg b.w.) at the time when all other animals received the last dose of the test article or test article vehicle. Test and control groups contained 40 or 20 animals. Two weeks after the last initiating dose, TPA in acetone (1 ug/0.2 ml/animal) or acetone only (0.2 ml) was applied topically to the shaved backskin of the animals three times a week for 20 weeks. The positive control group receiving urethane was treated similarly but with TPA only. The experimental design of these studies is summarized in Table 5-4.

All animals were held for 30 weeks following the first dose of initiator and then sacrificed. All test animals were necropsied and gross lesions evaluated histopathologically, as described below.

5.4.2 Experimental Observations

Animal Weights--

All animals were weighed at the initiation of the assay (6-8 weeks of age), weekly for the first 6 experimental weeks, and every four weeks thereafter.

Health Status Checks--

During the course of the assay all animals were checked twice daily on weekdays and once daily on weekends and holidays for mortality and moribundity. All animals found dead, sacrificed due to moribundity, or sacrificed at test termination were necropsied as described later.

Tumor Counts--

Starting weekly (at the time of the first topical application of promoter or promoter vehicle), the number, type, and location of each backskin tumor was recorded on an individual animal basis on Graphic Skin Tumor Data Sheets (Chart 5-6). Such tumors were classified as papillomas

Table 5-4 Experimental Design of Sencar Studies

MAI Study No.	Group	Dose[a]	Route[b]	Exposure (Initiation)	Promotion (TPA)	N[d]
1840.01	1	0.5 ml x 6	OR	Sample #1	Yes	40
	2	0.5 ml x 6	OR	Sample #1	No	20
	3	0.5 ml x 6	OR	Sample #2	Yes	40
	4	0.5 ml x 6	OR	Sample #2	No	20
	5	0.5 ml x 6	OR	Sample #3	Yes	40
	6	0.5 ml x 6	OR	Sample #3	No	20
	19	0.5 ml x 6	OR	2% Emulphor[c]	Yes	40
	20	0.5 ml x 6	OR	2% Emulphor	No	40
	21	500 mg/kg x 1	OR	Urethane	Yes	20
1840.03	7	0.5 ml x 6	OR	Sample #4	Yes	40
	8	0.5 ml x 6	OR	Sample #4	No	20
	9	0.5 ml x 6	OR	Sample #5	Yes	40
	10	0.5 ml x 6	OR	Sample #5	No	20
	11	0.5 ml x 6	OR	Sample #6	Yes	40
	12	0.5 ml x 6	OR	Sample #6	No	20
	22	0.5 ml x 6	OR	2% Emulphor	Yes	40
	23	0.5 ml x 6	OR	2% Emulphor	No	40
	24	500 mg/kg x 1	OR	Urethane	Yes	20
1840.04	13	0.5 ml x 6	OR	Sample #7	Yes	40
	14	0.5 ml x 6	OR	Sample #7	No	20
	15	0.5 ml x 6	OR	Sample #8	Yes	40
	16	0.5 ml x 6	OR	Sample #8	No	20
	17	0.5 ml x 6	OR	Sample #9	Yes	40
	18	0.5 ml x 6	OR	Sample #9	No	20
	25	0.5 ml x 6	OR	2% Emulphor	Yes	40
	26	0.5 ml x 6	OR	2% Emulphor	No	40
	27	500 mg/kg x 1	OR	Urethane	Yes	20

[a] Indicated doses were given 3 times a week for 2 weeks, except for urethane which was given on the final day of initiating (i.e., the 6th dosing day). Two weeks following the last initiating dose, promotion started with TPA being administered topically to the shaved back at 1.0 ug per animal in 0.2 ml acetone three times weekly for 20 weeks. Animals not receiving TPA got 0.2 ml acetone only for the same duration. Urethane was given in deionized water.

[b] OR = intragastric intubation.

[c] 2% Emulphor in distilled water

[d] Number of animals started on test.

Chart 5-6 Sencar Skin Tumor Data Sheet

Exposure: ____________________

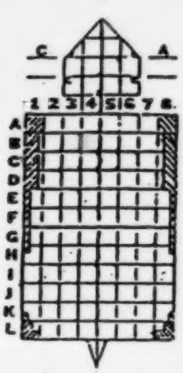
Comments: ____________________

Promotion:
TPA __________

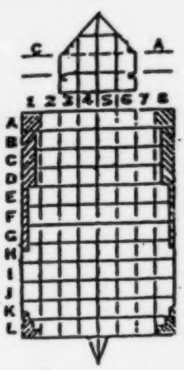
Acetone __________

Animal No. __________

Group No. __________


C
A
1 2 3 4 5 6 7 8
A B C D E F G H I J K L

d.__________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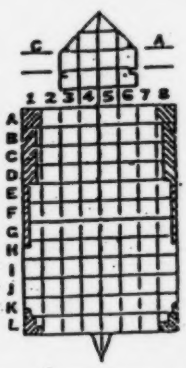
1 2 3 4 5 6 7 8
A B C D E F G H I J K L

d.__________


C
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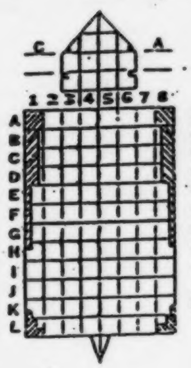
1 2 3 4 5 6 7 8
A B C D E F G H I J K L

d.__________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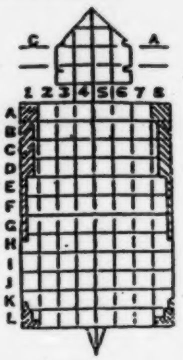
A B C D E F G H I J K L

d.__________


C
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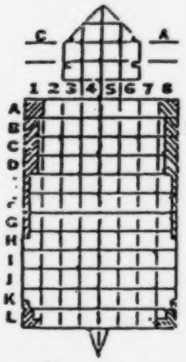
1 2 3 4 5 6 7 8
A B C D E F G H I J K L

d.__________


C
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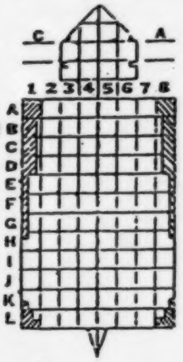
1 2 3 4 5 6 7 8
A B C D E F G H I J K L

d.__________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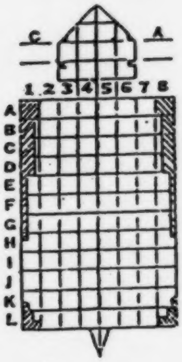
A B C D E F G H I J K L

d.__________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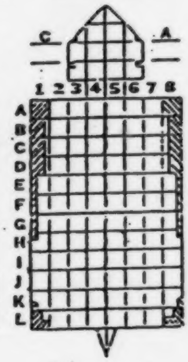
A B C D E F G H I J K L

d.__________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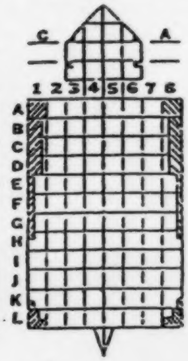
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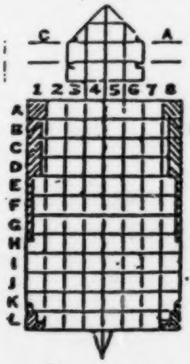
A B C D E F G H I J K L

d.__________


C
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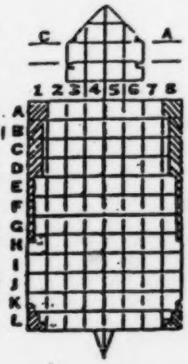
d.__________


C
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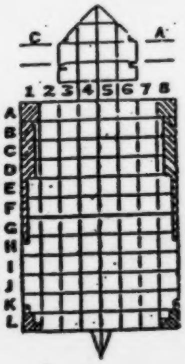
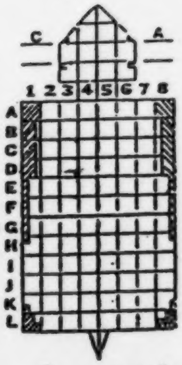
1 2 3 4 5 6 7 8
A B C D E F G H I J K L

d.__________


C
A
1 2 3 4 5 6 7 8
A B C D E F G H I J K L

d.__________


C
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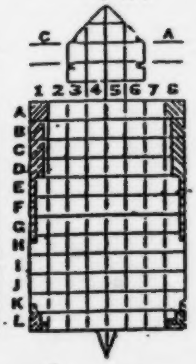
1 2 3 4 5 6 7 8
A B C D E F G H I J K L

d.__________


C
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1 2 3 4 5 6 7 8
A B C D E F G H I J K L

d.__________

or carcinomas, and whether or not they were cumulative tumors (persistent for three consecutive weeks) or actual tumors (present for any length of time).

Necropsy--

A complete and thorough necropsy was performed by MAI on each animal found dead or sacrificed during the course of the study. Gross observations on all external and internal lesions were recorded on Individual Necropsy Data Sheets for each animal including the number of skin papillomas and carcinomas observed. Each necropsy sheet and individual jar used to preserve the tissues of the animals was identified with the date of death or sacrifice, and indication of death or sacrifice. Photographs (slides) were taken of all gross skin tumors observed and the photos identified by the inclusion of the study, group, and animal number in the photographed field.

The following tissues were routinely examined and preserved in 10% neutral buffered formalin and their normality or gross pathology indicated and described on the Necropsy Data Sheet.

Lungs with trachea and thyroids
Liver
Kidneys and adrenals
Spleen
Pancreas
Heart
Brain
Muscle (from femur)
Lymph nodes (mesenteric, tracheo-
bronchial, subcutaneous)
Femur
Stomach
Duodenum (2" section below pylorus)
Colon and cecum
Ovaries, uterus, and cervix
Dorsal backskin
All other gross lesions

Histopathology--

Representative 5u Hematoxylin and Eosin stained slides were produced from paraffin embedded blocks of each gross lesion found at necropsy. All the residual tissues remaining from each gross lesion were bagged on an individual animal basis and retained. Slides were prepared and subsequently examined and evaluated by a veterinary pathologist at PAI.

5.4.3 Presentation and Analysis of Data

Information recorded on the Individual Graphic Skin Tumor Data Sheets was used to generate a data presentation on computer disc files. The data listed in Table 5-5 were collected at the frequency indicated both on an

Table 5-5 Data Presentation on Computer Disc Files

Data	Data Code	File No.	Range of Data	Data Units in the Range
Survival (wk 4-30)	S	4004DAT1	D1..AD281	1 = alive
Actual Papillomas (wk 4-30)	AP	4004DAT1	AE1..BE281	# of papillomas
Actual Carcinomas (wk 4-30)	AC	4004DAT1	BF1..CF281	# of carcinomas
Animal Number	AN#	4004DAT1	A1..A281	animal number
Group Number	GP#	4004DAT1	B1..B291	group number
No. Animals on Test	#S	4004DAT1	C282..C291	# started
No. Survivors/grp (wk 4-30)	SN	4004DAT1	D282..AD291	# of survivors
Percent Survival of animals promoted/grp (wk 4-30)	SP	4004DAT1	AE282..BE291	percent
Percent Survivors with actual skin tumors/grp (wk 4-30)	PATU	400DAT1	BF282..CF291	percent
Actual Skin Tumors per surviving animal/grp (wk 4-30)	ATPA	4004DAT1	CG282..DG291	# of tumors
Cumulative Papillomas (wk 4-30)	CP	4004DAT2	D1..AD281	# of papillomas
Cumulative Carcinomas (wk 4-30)	CC	4004DAT2	AE1..BE281	# of carcinomas
Weight (wk 0-30)	W	4004DAT2	BF1..BR281	weight (g)
Animal Number	AN#	4004DAT2	A1..A281	animal #
Group Number	GP#	4004DAT2	B1..B291	group #
No. Animals on Test	#S	4004DAT2	C282..C291	# started
Percent of Promoted Animals w/cumulative skin tumors/grp (wk 4-30)	PCTA	4004DAT2	D282..AD291	percent
Total Cumulative Skin Tumors/grp (wk 4-30)	CT	4004DAT2	AE282..BE291	# of cum tumors
Number of Cumulative Skin Tumors/animal promoted per grp (wk 4-30)	CTPA	4004DAT2	BF282..CF291	# of cum tumors per animal
Average Weight per animal per group (wk 0-30)	AW	4004DAT2	CG282..CS291	weight (g)
Standard Deviation of average wt animal per group (wk 0-30)	SDW	4004DAT2	CT282..DF291	± weight (g)

individual animal and on a group basis and entered onto a Lotus 1-2-3 program using an IBM XT Personal Computer with 384K memory.

Using the schemata shown in Table 5-5, the following parameters of skin tumor formation were compared between groups:

1. Percent of animals with one or more cumulative skin tumors.

2. The mean number of cumulative skin tumors per animal promoted.

3. The time to first scoring of one or more cumulative skin tumors per group.

4. The time to reach a cumulative skin tumor incidence of 50%.

The initiating potential of each test article evaluated was determined based on a comparison of the above parameters in test groups vs. appropriate controls, and a demonstration of statistical significance for the observed differences. Criteria for a valid test included a significant difference between the positive control group (Urethane initiation and TPA promotion) and each control group (vehicle initiation with vehicle promotion and vehicle initiation with TPA promotion). Each group receiving a test article as initiator and TPA promotion was compared to appropriate solvent controls (vehicle initiation and TPA promotion) to determine the significance of differences observed and attributable to the administration of test article as an initiator. Each group receiving test article as initiator followed by TPA promotion was also compared to groups receiving test articles as initiator and vehicle during promotion to determine if any observed induction of skin tumors was attributable to a complete carcinogenic potential of the test article.

5.4.4 Records

All records pertaining to these studies were retained in the Archives at the location in which they were generated - either MAI or PAI.

5.5 RAT LIVER FOCUS ASSAY

5.5.1 Test Parameters

For each study, male Sprague-Dawley rats were subjected to two-thirds partial hepatectomies and then dosed once intragastrically with either 3 ml of test sample (undiluted, prepared as previously described in Sections 5.1.2 and 5.1.4), 3 ml of test sample vehicle (2% Emulphor), or positive control (DEN, 50 mg/kg body weight) precisely 24 hours after the hepatectomies. Each test or control group contained 10 animals. Seven to nine days after the partial hepatectomies, all rats received 0.05% phenobarbitol (PB) in their drinking water for a period of 8 weeks after which they were held for an additional week on drinking water without PB and sacrificed. Cryotomy sections from the livers of the animals were then stained for histochemical demonstration of GGT positive foci.

5.5.2 Experimental Observations

Each of the four liver slides prepared from each test animal were microscopically evaluated for:

- the number of GGT+ foci with 9 or more nuclei per liver section
- the total number of morphometry points over areas of GGT+ Zone 1 induction
- the total number of morphometry points over the section of liver evaluated
- The radius in cm of each GGT+ focus with 9 or more nuclei

Findings were recorded on Slide Evaluation Sheets.

5.5.3 Analyses and Presentation of Data

Using the raw data from the Slide Evaluation Sheets, an Animal Summary Sheet was prepared for each test group, containing the following calculated data for each animal:

- the area of GGT+ Zone 1 Induction (ZI) in cm^2
- the area of liver evaluated in cm^2
- the percent of liver evaluated exhibiting ZI
- the number of GGT+ foci scored/cm^2
- the number of GGT+ foci scored/cm^3

Group means and standard errors of the means (SEM) were calculated for the percent of liver evaluated exhibiting ZI, the number of GGT+ foci/cm^2 and the number of GGT+ foci/cm^3.

Again using the raw data from the Slide Evaluation Sheets, a GGTase Positive Foci Area Summary Sheet was prepared for each test group, containing the following calculated data for each animal:

- the individual area in cm^2 for each scored GGT+ focus
- the total GGT+ focus area in cm^2 per animal
- the percent of liver evaluated exhibiting scored GGT+ foci

The group means and SEM for GGT+ focus areas were presented together with the total number of foci per group. The group mean percent of liver evaluated exhibiting GGT+ foci were also presented together with the sample size (number of animals evaluated with more than 2 cm^2 of liver sections) and the SEM.

To summarize the study results, Group Summary Sheets were prepared containing the following information for each of the test groups:

- the mean GGT+ focus area for each group with appropriate data concerning the number of GGT+ foci with 9 more nuclei scored per group and the SEM.
- the mean number of GGT+ foci scored per cm^2 of liver evaluated for each group with appropriate data concerning the number of animals evaluated and the SEM.
- the mean number of GGT+ foci scored per cm^3 of liver evaluated for each group with appropriate data concerning the number of animals evaluated and the SEM.
- the group mean percent of liver evaluated exhibiting GGT+ zone 1 induction with appropriate data concerning the number of animals evaluated and the SEM.
- the group mean percent of liver evaluated exhibiting GGT+ foci with appropriate data concerning the number of animals evaluated and the SEM.

A two-tailed *t*-test, for the comparison of two means from populations that are not known to have the same variance, was then used to evaluate the data parameters on the Group Summary Sheet (described above).

5.5.4 Records

All records pertaining to these studies were retained in the Archives at the location in which they were generated - either MAI or PAI.

5.6 LUNG ADENOMA ASSAY IN STRAIN A MICE

5.6.1 Test Parameters

For each study, A/J Strain mice (approximately 8 weeks of age) were dosed intragastrically with 0.25 ml of test sample or sample vehicle (2% Emulphor) 3X per week for 8 weeks. A positive control group received a single *per os* administration of urethane (10mg/mouse). Two dose groups, receiving 1X and 1/2X or 1/2X and 1/4X sample concentrations (prepared as previously described in Sections 5.1.2 and 5.1.4), were employed per test sample. In addition to the vehicle and positive control groups, an untreated control group was also included to determine the incidence of spontaneous lung tumors. Each test or control group contained a total of 40 mice (20 male and 20 female). Following completion of the 8 week treatment period, mice were held for 16 weeks and then sacrificed and necropsied at the end of the 24th week when they were approximately 32 weeks of age.

5.6.2 Experimental Observations

Body Weights--

Body weights of all mice were determined once every two weeks during the course of the study.

Health Status Checks--

Cage-side observations of all animals were performed twice-daily to note any dead or moribund animals and detailed health checks were performed once every two weeks at the time of weighing.

Necropsy and Histopathology--

At the end of the 24th experimental week (when mice were 32 weeks of age), all mice were weighed, sacrificed by CO_2 overdose, and subjected to gross necropsy. Any animals found moribund or dead were also necropsied, except for those animals where pathological examination was precluded due to severe post mortem autolysis. Gross necropsy findings for all animals were entered in a necropsy log book (with an entry of "dead" indicative of severe post mortem autolysis).

In the first study (ETOX-84-1001-02), photographs were taken of lung adenomas of positive control females during gross necropsy using a 35 mm camera. These photographs were processed and when reviewed the adenomas were not identifiable. Photographs were not taken of the remaining animals. In the second study (ETOX-84-1001-04), color micrographs of all grossly observed nodules were taken with a 35 mm camera.

Lung adenoma counts were made for each animal necropsied. Lungs were perfused by tracheal cannulation and placed in separate labelled vials for subsequent counting of the lung adenomas. Buffered formalin (10%) was used as the perfusate and fixative. Individual lung lobes were observed under a dissecting microscope and adenoma counts recorded on forms depicting the individual lung lobes (Chart 5-7). Dual counts were made by two technicians working independently of each other's observations. Differences between the two observers were reconciled by a third independent count. Randomly selected tumor-bearing lungs from each group (10%) were sent to Pathology Associates, Inc. for histological confirmation of adenomas and adenoma counts.

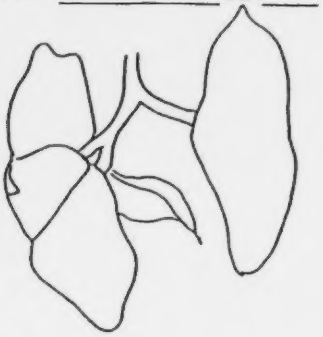
5.6.3 Analysis and Presentation of Data

In the first presentation, using the individual biweekly body weights generated over the test period, a mean, standard deviation, and standard error was calculated for the male and female mice in each test or control group. In the second presentation using the body weights at sacrifice and the number of lung adenomas subsequently counted for each individual, a mean, standard deviation, and standard error was calculated for the male and female mice in each test or control group.

Chart 5-7 Lung Adenoma Counting Form

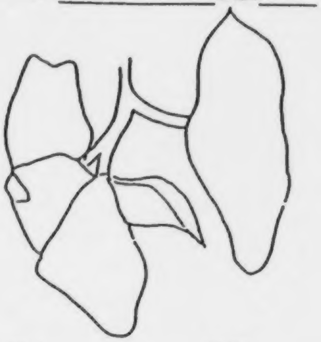
ENVIRONMENTAL HEALTH RESEARCH AND TESTING, INC., ENVIRONMENTAL TOXICOLOGY DIV.
3235 OMNI DRIVE, CINCINNATI, OHIO 45245
MURINE LUNG-ADENOMA STUDY NO.: ______________________________

MICE NO.: ______________ CAGE ____



____ COUNTS ____

MICE NO.: ______________ CAGE ____



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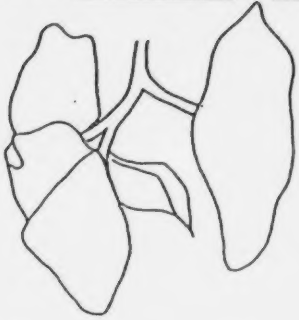
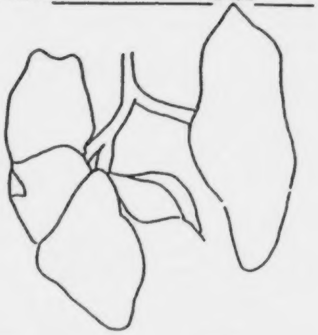
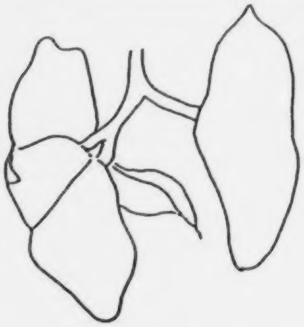


____ COUNTS ____

MICE NO.: ______________ CAGE ____

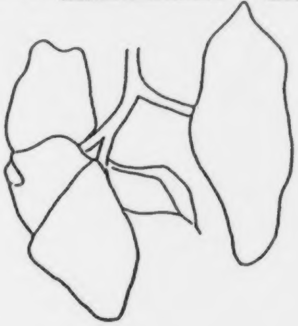


MICE NO.: ______________ CAGE ____



____ COUNTS ____

MICE NO.: ______________ CAGE ____



5.6.4 Records

All records pertaining to these studies including the raw data and other materials as well as a copy of the final and interim reports were retained and placed in the EHRT Archives at the conclusion of the study.

SECTION 6

RESULTS AND DISCUSSION

6.1 *IN VIVO* TOXICITY TESTS

6.1.1 Eight Sample Series (Study 1, Feb/March 1984)

The mean body weights and organ weights for the male and female mice in each test or control group are presented in Tables 6-1, 6-2 and 6-3. These data indicate that none of the eight samples produced any overt toxic effects in the test groups, at either concentration, when compared to the control group. In agreement with these findings, results of the detailed health status checks revealed no observable toxic effects (included as raw data with the Assay Report package).

The mean weekly water consumption per animal for each test or control group is presented in Table 6-4.

6.1.2 Five Sample Series (Study 2, Oct/Dec 1984)

The mean body weights and organ weights for the male and female mice in each test or control group are presented in Tables 6-5, 6-6 and 6-7. These data indicate that none of the five samples produced any overt toxic effects in the test groups, at either concentration, when compared to the control group. In agreement with these findings, results of the detailed health status checks revealed no observable toxic effects (included as raw data with the Assay Report package).

6.1.3 Submission of Study Materials

All residual quantities of samples, animal health records, and preserved tissue samples were submitted to the sponsor at or prior to submission of this Final Report.

Table 6-1 Effects of the Eight Sample Series: Mean Body Weights of Male and Female Mice During the 30-Day Exposure Period

		Mean Body Weights (g)									
		Males					Females				
Sample ID	Concentration	Day 1	Day 7	Day 14	Day 21	Day 28	Day 1	Day 7	Day 14	Day 21	Day 28
Control (deionized water)	-	30.1	31.7	33.0	35.5	35.0	22.1	23.8	24.7	26.9	26.5
1-6L-P74	1/4X	31.2	32.8	34.6	36.3	36.5	20.3	22.2	23.4	25.7	25.4
	1X	30.6	32.7	34.3	36.4	36.2	21.0	23.0	24.2	26.1	26.2
2-6L-P76	1/4X	29.5	31.3	32.9	35.3	34.7	21.2	22.7	23.9	25.8	25.6
	1X	30.5	31.5	32.5	35.3	35.2	22.3	24.5	26.5	27.6	27.8
3-6L-P78	1/4X	30.6	32.5	34.0	35.7	36.1	22.4	24.0	25.5	26.5	26.3
	1X	29.4	29.8	32.2	34.7	34.1	20.6	22.3	23.8	25.4	25.7
4-6L-P80	1/4X	30.1	31.2	32.5	35.4	35.1	22.4	24.1	25.7	27.3	26.9
	1X	30.1	30.6	34.5	36.1	36.3	20.8	22.3	23.8	25.4	25.9
5-6L-P82	1/4X	29.3	31.3	32.8	34.9	35.2	21.1	22.7	24.0	26.1	26.3
	1X	28.5	30.3	32.3	34.2	33.9	21.7	23.6	25.1	26.1	26.5
6B-2G-B-1+B-2-P68	1/10X	30.4	32.1	34.0	35.7	36.2	23.3	24.5	25.8	27.5	29.0
6A-2G-A-1+A-2-P70	1X	29.8	31.4	32.8	35.0	35.1	20.6	22.2	23.2	24.7	24.8
7B-2G-B-1+B-2-P64	1/10X	30.4	32.2	33.8	35.9	35.9	21.7	22.8	23.8	25.4	25.6
7A-2G-A-1+A-2-P66	1X	30.0	31.7	33.3	35.5	36.2	21.2	23.0	23.9	25.6	25.5
8B-2G-B-1+B-2-P60	1/10X	29.7	31.7	33.4	35.7	35.6	20.3	22.0	23.2	24.5	24.9
8A-2G-A-1+A-2-P62	1X	30.4	32.4	33.1	36.7	35.7	20.8	23.4	24.6	26.6	26.7

Table 6-2 Effects of the Eight Sample Series: Mean Body and Organ Weights of Male Mice at Necropsy

		Mean Body, Organ Weights (g)						
Sample ID	Concentration	Body	Liver	Brain	Spleen	Lungs	Kidneys	Testes
Control (deionized water)	-	35.8	2.477	0.472	0.133	0.382	0.664	0.295
1-6L-P74	1/4X	37.2	2.301	0.515	0.170	0.392	0.730	0.297
	1X	37.2	2.548	0.521	0.129	0.321	0.799	0.328
2-6L-P76	1/4X	35.2	2.301	0.521	0.168	0.354	0.763	0.331
	1X	35.7	2.234	0.510	0.134	0.315	0.729	0.303
3-6L-P78	1/4X	36.4	2.235	0.521	0.127	0.371	0.711	0.303
	1X	34.9	2.221	0.460	0.158	0.287	0.712	0.276
4-6L-P80	1/4X	37.5	2.764	0.518	0.152	0.299	0.739	0.319
	1X	36.8	2.251	0.455	0.115	0.308	0.675	0.276
5-6L-P82	1/4X	36.4	2.433	0.529	0.122	0.280	0.715	0.304
	1X	36.9	2.457	0.448	0.083	0.243	0.686	0.254
6B-2G-B-1+B-2-P68	1/10X	37.0	2.387	0.515	0.139	0.296	0.705	0.281
6A-2G-A-1+A-2-P70	1X	36.1	2.489	0.478	0.139	0.309	0.696	0.294
7B-2G-B-1+B-2-P64	1/10X	36.5	2.246	0.509	0.139	0.314	0.732	0.338
7A-2G-A-1+A-2-P66	1X	37.9	2.322	0.519	0.129	0.293	0.691	0.297
8B-2G-B-1+B-2-P60	1/10X	36.0	2.187	0.507	0.149	0.330	0.716	0.339
8A-2G-A-1+A-2-P62	1X	36.3	2.295	0.483	0.151	0.318	0.730	0.305

Table 6-3 Effects of Eight Sample Series: Mean Body and Organ Weights of Female Mice at Necropsy

		Mean Body, Organ Weights (g)						
Sample ID	Concentration	Body	Liver	Brain	Spleen	Lungs	Kidneys	Ovaries
Control (deionized water)	-	27.4	1.674	0.508	0.143	0.288	0.460	0.048
1-6L-P74	1/4X	25.5	1.546	0.450	0.105	0.212	0.430	0.026
	1X	27.6	1.630	0.480	0.123	0.244	0.476	0.026
2-6L-P76	1/4X	27.0	1.665	0.450	0.176	0.269	0.417	0.014
	1X	29.0	1.859	0.482	0.101	0.254	0.468	0.027
3-6L-P78	1/4X	28.0	1.768	0.436	0.096	0.260	0.428	0.017
	1X	26.6	1.563	0.480	0.115	0.233	0.440	0.027
4-6L-P80	1/4X	27.9	1.639	0.404	0.069	0.171	0.382	0.060
	1X	26.2	1.717	0.478	0.139	0.255	0.487	0.076
5-6L-P82	1/4X	26.7	1.568	0.452	0.118	0.233	0.427	0.039
	1X	27.6	1.817	0.484	0.137	0.252	0.498	0.048
6B-2G-B-1+B-2-P68	1/10X	28.2	1.708	0.486	0.121	0.259	0.464	0.048
6A-2G-A-1+A-2-P70	1X	25.3	1.538	0.476	0.103	0.247	0.429	0.032
7B-2G-B-1+B-2-P64	1/10X	27.6	1.732	0.450	0.143	0.266	0.470	0.039
7A-2G-A-1+A-2-P66	1X	25.8	1.708	0.467	0.108	0.269	0.482	0.044
8B-2G-B-1+B-2-P60	1/10X	25.9	1.515	0.461	0.109	0.235	0.420	0.040
8A-2G-A-1+A-2-P62	1X	27.6	1.693	0.456	0.134	0.258	0.449	0.046

Table 6-4 Effects of the Eight Sample Series: Water Consumption During the 30-Day Exposure Period

		Mean Weekly Water Consumption Per Animal (g)			
Sample ID	Concentration	Week 1	Week 2	Week 3	Week 4
Control (deionized water)	-	82.7	51.9	50.1	55.6
1-6L-P74	1/4X	59.5	47.7	45.9	46.6
	1X	55.3	58.8	59.2	53.2
2-6L-P76	1/4X	64.6	55.4	52.5	54.9
	1X	75.1	74.2	69.5	70.5
3-6L-P78	1/4X	59.8	52.0	50.4	52.4
	1X	61.8	67.1	60.3	63.2
4-6L-P80	1/4X	65.4	59.7	55.9	59.7
	1X	69.2	69.4	64.9	66.3
5-6L-P82	1/4X	55.0	54.1	48.5	48.5
	1X	65.3	62.2	60.4	59.1
6B-2G-B-1+B-2-P68	1/10X	57.0	54.9	54.9	55.2
6A-2G-A-1+A-2-P70	1X	65.7	60.5	62.0	66.4
7B-2G-B-1+B-2-P64	1/10X	53.6	50.5	51.6	52.3
7A-2G-A-1+A-2-P66	1X	54.4	59.1	58.7	58.5
8B-2G-B-1+B-2-P60	1/10X	36.3	53.0	53.0	53.6
8A-2G-A-1+A-2-P62	1X	45.1	63.9	62.8	67.1

Table 6-5 Effects of the Five Sample Series: Mean Body Weights of Male and Female Mice During the 30-Day Exposure Period

		Mean Body Weights (g)									
		Males					Females				
Sample ID	Concentration	Day 1	Day 7	Day 14	Day 21	Day 28	Day 1	Day 7	Day 14	Day 21	Day 28
Control (2% Emulphor)	-	31.3	33.2	35.1	37.1	38.3	27.3	27.7	29.1	31.4	29.8
1-9/84	1/4X	31.9	31.2	32.8	34.4	35.2	26.9	27.5	27.4	27.9	27.7
	1X	30.8	30.3	33.1	34.7	35.3	26.9	26.6	27.6	28.0	28.7
2-9/84	1/4X	30.9	31.2	32.4	35.2	35.8	26.7	27.1	28.1	28.5	28.3
	1X	30.9	30.6	32.8	34.7	35.9	26.1	26.2	27.7	27.5	28.1
3-9/84	1/4X	32.2	31.2	34.1	34.9	36.1	26.6	26.9	27.8	28.3	28.6
	1X	30.5	31.0	33.3	34.0	35.4	26.9	27.2	28.0	28.1	27.9
4-9/84	1/4X	33.0	33.3	35.4	37.2	37.8	26.4	26.8	27.5	27.9	28.1
	1X	31.6	32.0	33.9	35.1	36.3	26.9	26.9	27.6	27.7	28.3
5-9/84	1/4X	32.4	32.7	34.0	35.3	36.8	27.7	27.6	29.1	27.9	29.0
	1X	31.1	29.7	31.1	34.3	35.5	27.4	26.8	27.5	27.6	28.2

Table 6-6 Effects of the Five Sample Series: Mean Body and Organ Weights of Male Mice at Necropsy

Sample ID	Concentration	Mean Body, Organ Weights (g)						
		Body	Liver	Brain	Spleen	Lungs	Kidneys	Testes
Control (2% Emulphor)	-	38.1	2.164	0.518	0.139	0.283	0.593	0.320
1-9/84	1/4X	35.0	1.823	0.506	0.140	0.243	0.586	0.296
	1X	34.1	1.737	0.499	0.142	0.243	0.563	0.248
2-9/84	1/4X	36.1	1.984	0.524	0.162	0.219	0.597	0.291
	1X	36.2	1.759	0.508	0.126	0.223	0.535	0.263
3-9/84	1/4X	35.2	1.655	0.518	0.139	0.258	0.586	0.308
	1X	34.5	1.470	0.469	0.157	0.249	0.495	0.270
4-9/84	1/4X	35.5	1.522	0.502	0.097	0.233	0.492	0.260
	1X	31.4	1.106	0.428	0.065	0.195	0.434	0.224
5-9/84	1/4X	29.6	2.184	0.482	0.150	0.275	0.553	0.275
	1X	35.5	1.982	0.421	0.103	0.205	0.528	0.242

Table 6-7 Effects of the Five Sample Series: Mean Body and Organ Weights of Female Mice at Necropsy

Sample ID	Concentration	Mean Body, Organ Weights (g)						
		Body	Liver	Brain	Spleen	Lungs	Kidneys	Ovaries
Control (2% Emulphor	-	29.9	1.538	0.522	0.122	0.220	0.381	0.055
1-9/84	1/4X	27.4	1.328	0.526	0.121	0.210	0.385	0.063
	1X	27.3	1.292	0.483	0.117	0.223	0.391	0.059
2-9/84	1/4X	28.6	1.425	0.499	0.126	0.220	0.378	0.063
	1X	28.6	1.309	0.500	0.146	0.232	0.385	0.064
3-9/84	1/4X	27.7	1.311	0.494	0.122	0.249	0.385	0.067
	1X	27.5	1.194	0.500	0.135	0.239	0.365	0.044
4-9/84	1/4X	28.3	1.220	0.429	0.082	0.195	0.318	0.016
	1X	27.3	1.165	0.456	0.104	0.220	0.333	0.032
5-9/84	1/4X	34.3	1.548	0.518	0.117	0.228	0.368	0.032
	1X	28.3	1.400	0.479	0.108	0.206	0.347	0.030

6.2 SALMONELLA TYPHIMURIUM MUTAGENESIS ASSAYS

6.2.1 Nine Sample Series

Sample Test Results--

The nine sample series was tested in five separate experiments. Experiments performed 2/2/84, 2/9/84, 4/6/84 (B series samples), 9/12/84, and 3/30/85 were designed to determine the stability of the concentrated drinking water samples (in 2% Emulphor) during the concurrent testing in the animal assays (Sencar, rat liver focus, and lung adenoma). Results of these experiments, perviously reported in Assay Reports, have been summarized and are presented in Tables 6-8 and 6-9. These summarized results indicate that there were no significant differences in mutagenic response between drinking water samples concentrated and stored in 2% Emulphor at 4°C for 62 weeks (3/30/85 assay), for 33 weeks (9/12/84 assay), for 11 weeks (4/6/84 assay), for 3 weeks (2/9/84 assay), and for 2 weeks (2/2/84 assay). The relative mutagenic potency of the samples has remained consistent; in that, sample 5 appears most mutagenic, sample 3 least mutagenic, and samples 9, 4, and 7 within the range created by these two samples. Samples 1, 2, 6, and 8 were negative in all assays.

Results from the first two experiments indicated: 1) that the presence of the S9 activating system decreased or eliminated the positive responses observed without S9 addition, and 2) that higher sample doses were necessary in order to generate dose responses curves for the positive samples. Accordingly, in all subsequent experiments, samples were tested without S9 activation and the sample dose range was raised from the initial 100, 50, 25, 12.5 ul range to a 200, 100, 50, 25 ul range for all samples except the weakly mutagenic sample 3. The dose range for sample 3 was raised to 400, 200, 100, and 50 ul.

The experiment performed 4/6/84 (results summarized in Table 6-10) was designed to compare the stability of concentrated drinking water samples stored in 2% Emulphor for 11 weeks (B series) and those freshly concentrated from original acetone samples stored at -20°C for the same period (C Series). Similar mutagenic responses were observed between the two series, thus indicating that the mutagenic compounds in these samples are stable under two different storage conditions. It was also noted that the use of higher doses (described above) produced dose-dependent response curves for the positive samples.

QC Results--

For experiments 2/2/84, 2/9/84, and 4/6/84, no sample toxicity was observed and results of sterility testing indicated that all solutions were sterile. Negative solvent control numbers were within established ranges, except for the TA100 controls in the 2/9/84 experiment which were somewhat higher than expected. Control chemicals 2-nitrofluorene, sodium azide, and 2-anthramine demonstrated positive dose-related increases. However, total revertant numbers obtained with the 2-anthramine controls in the 2/2/84 experiment were unusually low.

Table 6-8 Stability Study of Nine Sample Series: Mutagenic Activity in TA98

Assay Date:	2/2/84		2/9/84		4/6/84	9/12/84	3/30/85
S9 Activation:	0	+	0	+	0	0	0
Samples Tested^{a}							
1-5L-P86	-(100)	-(100)	-(100)	-(100)	-(200)	-^{b}	-(200)
2-5L-P88	-(100)	-(100)	-(100)	-(100)	-(200)	-(200)	-(200)
3-5L-P90	+(50)	-(100)	+(50)	-(100)	±(200)	+(50)D	+(200)^{c}
4-5L-P92	+(100)D	-(100)	±(100)D	-(100)	+(100)D	+(25)D	+(25)D^{c}
5-5L-P94	+(50)D	-(100)	+(50)D	+(100)D	+(50)D	+(25)D	+(25)D^{c}
6-5L-P96	-(100)	-(100)	-(100)	-(100)	-(200)	-(200)	-(200)
7-5L-P98	+(100)D	-(100)	±(100)	-(100)	+(200)D	+(25)D	-(200)
8-5L-P30	-(100)	-(100)	-(100)	-(100)	±(200)	-^{b}	-(200)
9-5L-P38	+(25)D	-(100)	+(100)D	-(100)	+(50)D	+(25)D	+(100)

+ = revertant count two-fold or more above spontaneous background
± = dose-related increase but less than two-fold above background
D = dose response observed
( ) = value in parenthesis behind + or ± response is lowest dose (ul) at which positive response occurred; behind - response, the highest dose tested.

[a] All samples tested in this study were originally concentrated on 1/21/84 (See Sample Preparation)and stored at 4°C in 2% Emulphor.

[b] Although the plate count(s) was two-fold over the spontaneous count, the response was considered negative in the Assay Report, due to the unusually low spontaneous count for TA98 and the lack of a dose response curve.

[c] This result is based on a repeat assay (5/14/85) rather than the original 3/30/85 assay in which results for this sample were inconsistent with previous findings.

Table 6-9 Stability Study of Nine Sample Series: Mutagenic Activity in TA100

Assay Date	2/2/84		2/9/85		4/6/84	9/12/84	3/30/85
S9 Activation	0	+	0	+	0	0	0
Samples Tested[a]							
1-5L-P86	-(100)	-(100)	-(100)	-(100)	-(200)	-(200)	-(200)
2-5L-P88	-(100)	-(100)	-(100)	-(100)	-(200)	-(200)	-(200)
3-5L-P90	+(100)	-(100)	+(100)	-(100)	±(200)D	±(200)D	±(400)[b]
4-5L-P92	+(100)D	-(100)	+(100)D	-(100)	+(100)D	+(100)D	+(100)D[b]
5-5L-P94	+(25)D	+(100)D	+(50)D	+(100)D	+(50)D	+(25)D	+(25)D[b]
6-5L-P96	-(100)	-(100)	-(100)	-(100)	-(200)	-(200)	-(200)
7-5L-P98	+(100)D	-(100)	+(100)D	-(100)	+(200)D	+(100)D	+(200)D
8-5L-P30	-(100)	-(100)	-(100)	-(100)	+(200)	-(200)	-(200)
9-5L-P38	+(50)D	-(100)	+(100)D	-(100)	+(100)D	+(50)D	+(50)D

+ = revertant count two-fold or more above spontaneous background
± = dose-related increase but less than two-fold above background
D = dose response observed
( ) = value in parenthesis behind + or ± response is lowest dose (ul) at which positive response occurred; behind - response, the highest dose tested.

[a] All samples tested in this study were originally concentrated on 1/21/84 (See Sample Preparation) and stored at 4°C in 2% Emulphor.

[b] This result is based on a repeat assay (5/14/85) rather than the original 3/30/85 assay in which results for this sample were inconsistent with previous findings.

Table 6-10 Stability of Samples Stored in Emulphor (B Series) Vs Samples Stored in Acetone (C Series): Mutagenic Activity in TA98 and TA100

Assay Date: 4/6/84

S9 Activation: None

	B Series		C Series	
Strain:	TA98	TA100	TA98	TA100
Samples Tested				
1-5L-P86	-(200)	-(200)	-(200)	-(200)
2-5L-P88	-(200)	-(200)	-(200)	-(200)
3-5L-P90	±(200)	±(200)D	±(200)D	±(200)D
4-5L-P92	+(100)D	+(100)D	+(100)D	+(100)D
5-5L-P94	+(50)D	+(50)D	+(50)D	+(50)D
6-5L-P96	-(200)	-(200)	-(200)	-(200)
7-5L-P98	+(200)D	+(100)D	+(100)D	+(100)D
8-5L-P30	±(200)	-(200)	-(200)	-(200)
9-5L-P38	+(50)D	+(100)D	+(200)D	+(200)D

+ = revertant count two-fold or more above spontaneous background

± = dose-related increase but less than two-fold above background

D = dose response observed

( ) = value in parentheses behind + or ± response is lowest dose (ul) at which positive response occurred; behind - response, the highest dose tested

In the 9/12/84 and 3/30/85 experiments, no sample toxicity was observed and results of sterility testing indicated that all solutions were sterile. Control chemicals 2-nitrofluorene and sodium azide demonstrated positive dose-related increases. Negative solvent control numbers were within established ranges, but were uncharacteristically low for TA98 in the 9/12/84 experiment. During the initial performance of the 3/30/85 experiment, random contamination of some sample test plates was noted. Since all solutions employed tested out sterile, contamination was probably due to contaminated VBME plates or agar tubes. All samples having one or more contaminated plates were immediately retested (4/4/85) along with appropriate negative and positive controls.

6.2.2 Eighteen Sample Series

Sample Test Results

The eighteen sample series was evaluated for mutagenic activity in two separate experiments - 7/31/84 and 3/30/85. Results summarized from the Assay Reports are presented in Table 6-11. These results indicate that the samples positive in the 7/31/84 assay lost mutagenic activity over the 34 week storage period prior to the 3/30/85 retest. In the 7/31/84 assay in TA98, samples 11, 12, 17, 18, 19, 24 and 25 were positive. However in the 3/30/85 assay, sample 18 produced less than a 2-fold increase over background at the top two doses, and samples 17 and 25 were negative. Samples 10, 13, 14, 15, 16, 20, 21, 22, 23, 26, and 27 (-S9) were negative in both experiments. Sample 27 was positive in the 7/31/84 experiment with S9 activation but negative in the 3/30/85 retest. In the 7/31/84 assay in TA100, samples 12, 18, 19, 24, and 25 were positive. However, in the 3/30/85 assay, sample 25 produced less than a 2-fold increase over background at the top dose. Samples 10,11,13, 14, 15, 16, 20, 21, 22, 23, 26, and 27 were negative in both experiments. Sample 17 was also negative in both experiments although it did show a revertant count significantly above background at the 200 ul dose, in the 3/30/85 assay. Sample 27 with S9 addition was negative in both experiments.

Thus, in the 3/30/85 retest, there was a decrease in positive samples compared to the 7/31/84 results (from 7 to 4 for TA98 and from 5 to 4 for TA100) even though the sample dose range was increased from 100, 50, 25, 12.5 ul in the first experiment to 200, 100, 50, 25 ul for the retest.

In the 7/31/84 experiment, all positive samples except sample 27 exhibited a decreased response in the presence of the S9 activating system. Therefore, in the 3/30/85 retest all samples, except sample 27, were tested without activation.

QC Results--

In the 7/31/84 experiment, some sample toxicity was observed with sample 10 resulting in numerous phenocopies. Results of sterility testing indicated that all solutions were sterile. Control chemicals 2-nitrofluorene, sodium azide and 2-anthramine demonstrated positive dose-related increases. Negative solvent control numbers were generally

Table 6-11 Stability Study of Eighteen Sample Series: Mutagenic Response in TA98 and TA100

Assay Date:	7/31/84				3/30/85			
Strain:	TA98		TA100		TA98		TA100	
S9 Activation:	0	+	0	+	0	+	0	+
Samples Tested								
10-80ml-P32	- T	- T	- T	-(100)	-(200)		-(200)	
11-80ml-P34	+(25)D	-(100)	-(100)	-(100)	+(100)D		-(200)	
12-80ml-P36	+(25)D	+(50)D	+(100)D	-(100)	+(200)D		+(100)D	
13-80ml-P40	-(100)	-(100)	-(100)	-(100)	-(200)		-(200)	
14-80ml-P42	-(100)	-(100)	-(100)	-(100)	-(200)		-(200)	
15-80ml-P14	-(100)	-(100)	-(100)	-(100)	-(200)		-(200)	
16-80ml-P16	-(100)	-(100)	-(100)	-(100)	-(200)		-(200)	
17-80ml-P18	+(50)D	-(100)	-(100)	-(100)	-(200)		±(200)	
18-80ml-P20	+(50)D	-(100)	+(100)D	-(100)	±(100)D		+(100)D	
19-80ml-P22	+(25)D	-(100)	+(50)D	-(100)	+(200)D		+(50)D	
20-80ml-P24	-(100)	-(100)	-(100)	-(100)	-(200)		-(200)	
21-80ml-P26	-(100)	-(100)	-(100)	-(100)	-(200)		-(200)	
22-80ml-P46	-(100)	-(100)	-(100)	-(100)	-(200)		-(200)	
23-80ml-P48	-(100)	-(100)	-(100)	-(100)	-(200)		-(200)	
24-80ml-P50	+(25)D	+(50)D	+(100)D	-(100)	+(100)D		+(200)D	
25-80ml-P52	+(50)D	+(100)D	+(100)D	-(100)	-(200)		±(200)	
26-80ml-P54	-(100)	-(100)	-(100)	-(100)	-(200)		-(200)	
27-80ml-P56	-(100)	+(100)D	-(100)	-(100)	-(200)	-(200)	-(200)	-(200)

+ = revertant count 2-fold or more above spontaneous background
± = dose-related increase but less than 2-fold above background
D = dose response observed
T = toxicity observed, with phenocopies
( ) = value in parenthesis behind a + or ± response is the lowest dose (ul) at which positive response occurred; behind a - response, the highest dose tested

within established ranges, except for the TA100 solvent controls with S9 fraction which were lower than expected.

In the 3/30/85 experiment, no sample toxicity was observed and results of sterility testing indicated that all solutions were sterile. Control chemicals, sodium azide and 2-nitrofluorene, demonstrated positive dose-related increases. Results with the 2-anthramine controls yielded a positive result, but the revertant counts for the 1.0 and 2.0 ug doses were not dose-dependent. Negative solvent controls were within established ranges. During the performance of this experiment, random contamination of some sample test plates was noted. Since all solutions employed tested out sterile, contamination was probably due to contaminated VBME plates or agar tubes. All samples having one or more contaminated plates were retested (4/4/85) along with appropriate negative and positive controls.

6.2.3 Five Sample Series

Sample Test Results--

The five sample series was evaluated for mutagenic activity on 10/25/84. Results summarized from the Assay Report are presented in Table 6-12. As shown, samples 1 and 2 were negative; samples 3, 4, and 5 were positive with sample 3 the most mutagenic and sample 4 the least mutagenic. For all three positive samples, the presence of the S9 activating system decreased or negated the positive responses observed without S9 addition.

QC Results--

No sample toxicity was observed and results of sterility testing indicated that all solutions were sterile. Control chemicals sodium azide, 2-nitrofluorene, and 2-anthramine demonstrated positive dose-related increases. Negative solvent controls were within established ranges.

6.2.4 Submission of Study Materials

All residual quantities of samples were returned to the sponsor at the time this Final Report was submitted.

Table 6-12 Mutagenic Activity of Five Sample Series in TA98 and TA100

Assay Date:	10/25/84			
Strain:	TA98		TA100	
S9 Activation:	0	+	0	+
Samples Tested:				
1-9/84	-(200)	-(200)	-(200)	-(200)
2-9/84	-(200)	±(200)	-(200)	-(200)
3-9/84	+(50)D	+(200)D	+(100)D	± 200
4-9/84	+(200)D	-(200)	±(200)	-(200)
5-9/84	+(200)D	-(200)	+(200)D	-(200)

+ = revertant count two-fold or more above spontaneous background

± = dose-related increase but less than two-fold

D = dose response observed

( ) = value in parenthesis behind a + or ± response is the lowest dose (ul) at which a response occurred; behind - response, the highest dose tested.

6.3 SENCAR MOUSE SKIN BIOASSAY: INITIATION/PROMOTION

For reporting of Sencar results, treatment groups were identified as shown previously in Table 5-4, Section 5.4.1.

6.3.1 MAI Assay Report 1840.01 (Samples 1-5L-P86, 2-5L-P88, 3-5L-P90)

Sample Toxicity--

Of 280 animals started on test, 47 animals died or were sacrificed in moribund condition prior to the scheduled terminal sacrifice at 30 experimental weeks. Of those, 41 animals were lost during the two week initiation phase, of which 38 showed signs of intubation accidents (punctured esophagus or trachea, hemorrhagic lungs, or hemorrhage and inflammation of the esophagus). Three deaths not associated with overt signs of intubation trauma occurred in animals receiving test articles #1 (two animals) and #3 (one animal). Deaths and moribund sacrifices occurring after the initiation period and before the 30th experimental week were limited to 6 animals, two of which showed chronic problems associated with intubation trauma. The remaining four post-initiation losses were associated with malignant lymphoma (one in Group 3), anemia and liver inflammation (one in Group 5), and lymphopathy and/or TPA induced necrotic skin lesions (two in Group 5). Administration of the test compound per se did not appear to result in significant toxicity (see Data Worksheet Files, MAI Assay Report 1840.01).

When compared to vehicle control animals (Group 20), no significant differences in the mean body weights of other experimental groups were found during the course of the experiment except Group 6 at one and two experimental weeks (see Data Worksheet Files). The difference between Group 6 and the vehicle control was statistically significant by a two-tailed t-test (alpha =.05) at those times, but not at any other time point during the course of the assay. That difference may have been due to the transient effect of dosing with compound #3. In general the multiple administrations of the test articles did not result in permanently altered body weights during the course of the assay. These findings and the survival results described above indicated that the test compounds administered as described did not cause acute or delayed toxicities resulting in death or seriously altered body weights.

Gross Skin Tumor Data--

The gross scoring of skin tumors conducted during this assay proved to be quite accurate both in terms of the ability to detect a tumor and to classify them as papillomas or carcinomas. Histopathologic evaluations revealed only two cases where skin lesions scored grossly as papillomas were actually hyperplasias and one case (#284, Group 5) where a skin carcinoma was not scored grossly because it was masked by the ulcerative effects of TPA administration. The first animal with gross lesions not histologically confirmed as papillomas was #276 in Group 5, and the lesions were noted as atypical in the experimental records. Accordingly, the gross counts were eliminated and not included in reported data based on actual or cumulative papilloma counts. The second animal with a gross lesion not histologically confirmed as a papilloma was #312 in Group 19. Since it was not scored grossly until week 30 and fit the gross criteria for an early papilloma, it was included in the reported data. It therefore affected data based on actual counts, but not cumulative counts. Gross or microscopic skin carcinomas were observed on only two animals in the study (one tumor on each, animals received test articles 1 or 3 with TPA promotion). The lesion detected grossly was first scored as a carcinoma arising from an existing papilloma at week 30 in Group 1. The other was an incidental finding during histopathologic evaluation of Group 5, and was not reported in the gross data. The extremely small number of detected carcinomas and their late appearance eliminated the use of skin carcinoma formation as a parameter for evaluating the initiating potential of the test articles. The latency, incidence and multiplicity of cumulative skin papillomas (tumors persistent for three or more weekly tumor counts) were used as parameters for test evaluation.

The weekly count of actual skin tumors and cumulative skin tumors were recorded on the Lotus 1-2-3 worksheet files (see MAI Assay Report 1840.01) for each experimental group as were the percent incidence of animals with actual tumors or cumulative tumors, the total number of cumulative tumors, and the number of actual or cumulative tumors per surviving animal or animal promoted, respectively. Table 6-13 summarizes the results of the assay based on the designated data parameters .

All groups receiving test article initiation followed by acetone promotion showed no skin tumor formation, and the test articles as administered did not act as complete carcinogens for skin tumor formation. The control group receiving initiator and promoter vehicles (2% Emulphor and acetone) showed no skin tumor formation.

The positive control group receiving urethane initiation and TPA promotion showed a 65% incidence of animals with one or more cumulative papillomas by 30 experimental weeks and reached a 50% incidence at 26 experimental weeks. A total of 18 cumulative tumors were observed on 13 animals during the assay (0.90 cumulative tumors per effective animal or 1.38 cumulative tumors per cumulative tumor bearing animal). The first cumulative skin tumor was observed at 15 experimental weeks and the total number reached a maximum at 29 weeks. A total of 70% of the animals showed skin tumors during the course of the study although some were not persistent cumulative tumors.

The control group receiving initiator vehicle and TPA promotion showed the formation of skin tumors with a significantly weaker response than the positive control. The ability of Sencar mice to form skin tumors following TPA promotion in the absence of a known chemical initiator has been reproducibly demonstrated by several investigators. Nine percent of the effective animals in the group developed cumulative tumors by 30 experimental weeks. A total of three cumulative tumors were observed on three animals during the assay (0.09 cumulative tumors per effective animal or 1.0 cumulative tumors per cumulative tumor bearing animal). The first cumulative tumor was observed at 26 weeks (11 weeks after

Table 6-13 MAI Assay Report 1840.01 Cumulative Skin Tumor Data Summary

	Group and Treatment (Sample: Promoter)[a]								
Parameter	1 1:T	2 1:A	3 2:T	4 2:A	5 3:T	6 3:A	19 Ve:T	20 Ve:A	21 Ur:T
# of animals started on test	40	20	40	20	40	20	40	40	20
# of animals reaching the 1st Promotion	31	16	31	19	35	14	35	37	20
Effective # of animals (number alive at the time of the 1st cumulative skin tumor observed at 15 weeks in Grp. 21)	31	16	31	19	34[b]	14	35	36	20
# of animals surviving til term. sac. at 30 experimental weeks	31	16	30	19	32	14	35	36	20
# of cumulative skin tumors through 30 experimental weeks	5	0	5	0	5	0	3	0	18
# of animals with at least 1 cum. skin tumor through 30 experimental weeks	5	0	5	0	4	0	3	0	13
% effective animals with cum. skin tumors through 30 experimental weeks	16%	0%	16%	0%	12%	0%	9%	0%	65%
Cumulative skin tumors per effective animal	0.16	0	0.16	0	0.15	0	0.09	0	0.90
Cumulative skin tumors per cumulative skin tumor bearing animal	1.0	NA	1.0	NA	1.25	NA	1.0	NA	1.38
Time of 1st observed cumulative skin tumor (in experimental weeks)	19	NA	27	NA	19	NA	26	NA	15
Time of maximum cumulative skin tumor count (in experimental weeks)	28	NA	30	NA	30	NA	29	NA	29
Cumulative % animals promoted which showed skin tumors regardless if persistent for 3 weeks	19%	0%	23%	0%	29%	0%	17%	0%	70%

a Sample: Promoter Key: A = Acetone, T = TPA, Ve = Vehicle, Ur = Urethane

b Animal #276 eliminated since lesions scored grossly as papillomas were histologically identified as hyperplasias

NA = Not applicable

observation of the first cumulative tumor in the positive control group), and the total number reached a maximum at 29 experimental weeks. Only 17% of the group showed skin tumors during the course of the assay including those bearing tumors not persistent for three counting points.

Initiation with any of the test articles followed by promotion with TPA yielded tumor incidences in animals (cumulative skin tumors by 30 experimental weeks) that were not significantly different (Chi square, 0.05 level of significance) from the vehicle/TPA control. The assay itself was valid, since the incidence of cumulative skin tumors in the positive control group was statistically greater than the TPA control and the vehicle control groups (Emulphor/TPA and Emulphor/acetone) when analyzed by the Chi-square test. In fact, the number of cumulative skin tumors per effective animal and per cumulative tumor bearing animal were similar for all test article plus promotion groups and the promotion only control group (see Table 6-13). Time until a 50% incidence of cumulative tumors was not a valid parameter for comparison of the test articles to the positive control since the test article groups never attained higher than a 16% incidence. Although two of the test articles (#1 and #3) combined with TPA promotion resulted in a shorter latency period until first cumulative tumor appearance compared to the TPA only control, the effect was misleading. A more reasonable index based on latency is the average latency period for the appearance of all cumulative tumors on all tumor bearing animals within the group. Such an analysis indicated a mean latency (weeks) ± standard deviation for the appearance of all cumulative tumors of 23 ± 3.5, 29 ± 1.3, 26 ± 5 and 28 ± 1.5 for test articles #1, #2, #3, and TPA only, respectively. Thus, use of this latency index indicates that none of the test compounds caused a reduced latency compared to the TPA only control.

Histopathology Findings--

The results of the histopathological evaluation are presented in detail within the PAI Pathology Report (MAI Assay Report 1840.01 Appendix A) which contains the following data:

1) all diagnosed tissue changes together with the number of organs examined, summarized by group in a Project Summary Table for non-neoplastic and neoplastic lesions

2) neoplasms alone, summarized for the entire study in Summary Tumor Incidence

3) neoplasms alone, summarized by group in a Tumor Incidence Table

4) Individual Animal Data which includes microscopic observations by individual animal as well as gross to microscopic correlation where appropriate.

After review of the detailed data tables, the histopathology results were summarized by PAI as follows:

Early Deaths-- Out of a total of 280 animals on study, 131 animals were submitted for histopathology due to presence of grossly noted lesions at time of necropsy. Thirty-seven of these mice (see Table 6-14) were early deaths (before 14 days) due to gavage accident and/or acute toxicity. Differentiation between these two causes of death was not attempted. Lesions most commonly present in the early death mice included congestion of various organs, and occasionally esophageal or tracheal puncture. (Four additional early death mice -144, 148, 265, 362- had no tissues submitted for histopathology.)

Skin Neoplasms and Lesions-- Epithelial cell tumors occurred in mice from 5 of the 9 groups and in 32 of the 131 mice from which tissues were examined. Squamous cell carcinomas were present in two mice while squamous papillomas were diagnosed in 30 mice. The delineation between the benign papilloma and the malignant carcinoma was based principally on two criteria: (1) the degree of atypia of the neoplastic cells and; (2) the tendency of the basilar layer of epidermal cells to break through the basal lamina and infiltrate into the underlying dermis. A summary of the epithelial tumors is found in Table 6-14.

The largest number of skin tumors (18 squamous papillomas in 12 mice) occurred in the positive control (Group 21 - Urethane with promoter).

All three test samples, when given with the promoter, showed some degree of skin tumor development. Samples #1 and #3 with TPA promoter (Group 1 and 5) each had one squamous cell carcinoma as well as four (in four mice) and six (in five mice) squamous papillomas respectively. Additionally, Sample #3 with promoter had two animals with epithelial hyperplasia and three with skin ulceration. Sample #2 with promoter (Group 3) had six squamous papillomas (in five mice), as well as five mice with epithelial hyperplasia and four with skin ulcers. Sample #3 without promotion had one case of epithelial hyperplasia and one case of skin ulceration.

The promotion only control group tested with 2% Emulphor with TPA (Group 19) had four cases of squamous cell papilloma, five cases of epithelial hyperplasia, one case of ulceration, and four cases of chronic-active inflammation. The vehicle control group treated with 2% Emulphor and no promoter (Group 20) had no skin lesions.

Neoplasms in Organ Systems Other Than Skin-- Group 3 (Test Sample #2 with promoter) had one case of malignant lymphoma. Group 20 (carrier without promoter) had one uterine hemangioma. An endometrial stromal sarcoma occurred in one animal in Group 1 (sample #1 with promoter) and in one animal in Group 19 (carrier with promoter). Group 5 (sample #3 with promoter) had one pheochromocytoma. These tumors do not appear to be compound related.

Group 21 (urethane with promoter) had four mice with a total of eight alveolar/bronchiolar adenomas. These tumors may be compound related.

Table 6-14 Histopathology Findings: Skin Neoplasms and Lesions

Group	#Mice In Study	#Mice Sub. for Histo.	#Mice Dead by Day 14	#Mice With Sq. Cell Carcinoma	#Mice With Sq. Cell Papilloma	#Mice With Epithelial Hyperplasia	#Mice With Skin Ulcer
1	40	24	9*	1	4	0	0
2	20	8	4	0	0	0	0
3	40	21	9	0	5[a]	5	4
4	20	5	1	0	0	0	0
5	40	21	5**	1	5[a]	2	3
6	20	10	6	0	0	1	1
19	40	17	4	0	4	5	1
20	40	10	3**	0	0	0	0
21	20	15	0	0	12[b]	0	0

Two of these mice were not submitted for histo.
** One of these mice was not submitted for histo.

a - 6 sq. cell papillomas in 5 mice.
b - 18 sq. cell papillomas in 12 mice.

Lesions in Organ Systems Other Than Skin-- Remaining lesions were identified and tabulated. None appeared to be compound related or of other significance to warrant further comment.

Except for the induction of lung adenomas in the group treated with urethane (a result expected from previous experimentation), the formation of other non-neoplastic or neoplastic lesions was not observed to result from treatment with test articles alone or in combination with TPA. Skin ulceration associated with inflammation and hyperplasia were observed in various groups as expected and found to be caused by the administration of TPA.

Overall results of this study - including sample toxicity findings, gross skin tumor data, and histopathology findings - indicate that samples 1-5L-P86, 2-5L-P88, and 3-5L-P90 are not acutely toxic, are not complete carcinogens for tumor formation, and are not initiators of TPA promoted tumors of the skin or organ systems, when tested in the Sencar bioassay.

6.3.2 MAI Assay Report Study 1840.03 (Samples 4-5L-P92, 5-5L-P94,

Sample Toxicity--

Of 280 animals started on test, 12 animals died or were sacrificed in moribund condition prior to the scheduled terminal sacrifice at 30 experimental weeks. Of those, seven animals were lost during the two week initiation phase and all showed signs of intubation accidents (punctured esophagus or trachea, hemorrhagic lungs, or hemorrhage and inflammation of the esophagus). Deaths and moribund sacrifices occurring after the initiation period were limited to five animals, three of which showed malignant lymphomas (one in Group 7 and two in Group 9). The remaining two post-initiation losses were associated with lymphoid hyperplasia, glomerulasclerosis, or lymphoid infiltration found in one of the Group 12 animals and one of the Group 7 animals. Administration of the test compound per se did not appear to result in significant toxicity (see Data Worksheet Files, MAI Assay Report 1840.03).

When compared to vehicle control animals (Group 23), no significant differences in the mean body weights of other experimental groups were found during the first six weeks of the experimental period except for Group 22 (TPA promotion only control) at the 5th experimental week. The average body weight for Groups 7 (compound #4 plus TPA), 9 (compound #5 plus TPA), 11 (compound #6 plus TPA), and 22 (vehicle plus TPA) were significantly different from the control group at 22, 26 and 30 experimental weeks. In addition Group 7 was significantly different at weeks 10 and 18, and Group 8 (compound #4 plus acetone) was significantly different at 10 experimental weeks. The differences noted above were statistically significant by a two-tailed t-test (alpha = .05). These differences (except Group 8) seemed due to the effect of multiple TPA dosing as noted in Group 22. In general, the multiple administrations of the test articles did not result in prolonged alterations of body weights during the course of the assay. These findings and the survival results described above indicated that the test compounds administered as described did not cause acute or delayed toxicities resulting in death or altered body weights.

Gross Skin Tumor Data--

The gross scoring of skin tumors conducted during this assay proved to be quite accurate both in terms of the ability to detect a tumor and to classify them as papillomas or carcinomas. Histopathologic evaluations revealed only one case where a skin lesion scored grossly as a non-papillomatous plaque was actually a papilloma, and one case where a skin carcinoma was scored grossly as a papilloma. One skin carcinoma was observed on one animal receiving urethane with TPA promotion. The carcinoma, which was never scored as such grossly, arose from an existing papilloma, and was an incidental finding during histopathologic evaluation. The extremely small number of detected carcinomas and their late appearance in the assay eliminated the use of skin carcinoma formation as a parameter for evaluating the initiating potential of the test articles. The latency, incidence and multiplicity of cumulative skin papillomas (tumors persistent for three or more weekly tumor counts) were used as parameters for test evaluation.

The weekly count of actual skin tumors and cumulative skin tumors were recorded on the Lotus 1-2-3 worksheet files (See MAI Assay Report 1840.03) for each experimental group as were the percent incidence of animals with actual tumors or cumulative tumors, the total number of cumulative tumors, and the number of actual or cumulative tumors per surviving animal or animal promoted, respectively.

Table 6-15 summarizes the results of the assay based on the designated data parameters.

All groups receiving test article initiation with acetone promotion showed no skin tumor formation, and the test articles as administered did not act as complete carcinogens for skin tumor formation. The control group receiving initiator and promoter vehicles showed no skin tumor formation.

The positive control group receiving urethane initiation and TPA promotion demonstrated a 70% incidence of animals with one or more cumulative papillomas by 30 experimental weeks and reached a 50% incidence at 19 experimental weeks. A total of 28 cumulative tumors were observed on 14 animals during the assay (1.4 cumulative tumors per effective animal or 2.0 cumulative tumors per cumulative tumor bearing animal). The first cumulative skin tumor was observed at 13 experimental weeks and the total number reached a maximum at 30 weeks. A total of 70% of the animals showed skin tumors during the course of the study although some were not persistent cumulative tumors.

The control group receiving initiator vehicle and TPA promotion showed the formation of skin tumors with a significantly weaker response than the

Table 6-15 MAI Assay Report 1840.03 Cumulative Skin Tumor Data Summary

Parameter	Group and Treatment (Sample:Promoter)[a] 7 4:T	8 4:A	9 5:T	10 5:A	11 6:T	12 6:A	22 Ve:T	23 Ve:A	24 Ur:T
# of animals started on test	40	20	40	20	40	20	40	40	20
# of animals reaching the 1st Promotion	39	20	40	19	39	17	40	39	20
Effective # of animals (number alive at the time of the 1st cumulative skin tumor observed at 13 weeks in Grps 7&24)	38	20	40	19	39	17	40	39	20
# of animals surviving til term. sac. at 30 experimental weeks	37	20	39	19	39	16	40	39	20
# of cumulative skin tumors through 30 experimental weeks	6	0	3	0	13	0	10	0	28
# of animals with at least 1 cum. skin tumor through 30 experimental weeks	6	0	3	0	13	0	10	0	14
% effective animals with cum. skin tumors through 30 experimental weeks	16%	0%	8%	0%	33%	0%	25%	0%	70%
Cumulative skin tumors per effective animal	0.16	0	0.08	0	0.33	0	0.25	0	1.4
Cumulative skin tumors per cumulative skin tumor bearing animal	1.0	NA	1.0	NA	1.0	NA	1.0	NA	2.0
Time of 1st observed cumulative skin tumor (in experimental weeks)	13	NA	18	NA	18	NA	14	NA	13
Time of maximum cumulative skin tumor count (in experimental weeks)	21	NA	29	NA	28	NA	27	NA	30
Cumulative % animals promoted which showed skin tumors regardless if persistent for 3 weeks	23%	0%	23%	0%	38%	0%	35%	0%	70%

[a] Sample:Promoter Key: A = Acetone, T = TPA, Ve = Vehicle, Ur = Urethane
NA = Not applicable

positive control. The ability of Sencar mice to form skin tumors following TPA promotion in the absence of a known chemical initiator has been reproducibly demonstrated by several investigators. Twenty-five percent of the effective animals in the group developed cumulative tumors by 30 experimental weeks. A total of 10 cumulative tumors were observed on 10 animals during the assay (0.25 cumulative tumors per effective animal or 1.0 cumulative tumors per cumulative tumor bearing animal). The first cumulative tumor was observed one week after the positive control at 14 weeks, and the total number reached a maximum at 27 experimental weeks. Only 35% of the group showed skin tumors during the course of the assay including those bearing tumors not persistent for three counting points.

Initiation with test articles #4, #5 and #6, followed by promotion with TPA, yielded tumor incidences in animals (cumulative skin tumors by 30 experimental weeks) that were not significantly different (Chi-square, 0.05 level of significance) from the TPA only control. The assay itself was valid since the incidence of cumulative skin tumors in the positive control group was statistically greater than the TPA control and the vehicle control groups when analyzed by the Chi-square test. In fact the number of cumulative skin tumors per effective animal followed the same pattern, but the number of cumulative skin tumors per cumulative tumor bearing animal were similar for all test article plus promotion groups and the promotion only control group (See Table 6-15). The positive control group had twice as many cumulative skin tumors per cumulative tumor bearing animal than groups receiving the test articles plus TPA or TPA only. Time until a 50% incidence of cumulative tumors was not a valid parameter for comparison of the test articles to the positive control since the test article groups never attained higher than a 33% incidence. Although, two of the test articles (#5 and #6) combined with TPA promotion resulted in a longer latency period until first cumulative tumor appearance compared to the TPA only control, the effect was misleading. A more reasonable index based on latency is the average latency period for the appearance of all cumulative tumors on all tumor bearing animals within the group. Such an analysis indicated a mean latency (weeks) ± standard deviation for the appearance of all cumulative tumors of 17 ± 3.2, 25 ± 6.1, 22 + 3.4 and 22 ± 3.9 for test article #4, test article #5, test article #6, and TPA only, respectively. Use of this latency index indicates that none of the test compounds caused a significantly reduced latency compared to the TPA only control.

Histopathology Findings--

The results of the histopathological evaluation are presented in detail within the PAI Pathology Report (MAI Assay Report 1840.03 Appendix A) which contains the following data:

1) all diagnosed tissue changes together with the number of organs examined, summarized by group in a Project Summary Table for non-neoplastic and neoplastic lesions

2) neoplasms alone, summarized for the entire study in Summary Tumor Incidence

3) neoplasms alone, summarized by group in a Tumor Incidence Table

4) a table of Tabulated Animal Data, correlating diagnoses by organ and by individual animal within each dosage group

5) Individual Animal Data which includes microscopic observations by individual animal as well as gross to microscopic correlation where appropriate.

After review of the detailed data tables, the histopathology results were summarized by PAI as follows:

Skin Neoplasms and Lesions-- Epithelial cell tumors occurred in mice from five of the nine groups and in 42 of the 130 mice from which tissues were examined. A squamous cell carcinoma was present in one mouse (Group 24-Urethane with promoter) while squamous papillomas were diagnosed in all of the 42 mice. The delineation between the benign papilloma and the malignant carcinoma was based principally on two criteria: (1) the degree of atypia of the neoplastic cells and; (2) the tendency of the basilar layer of epidermal cells to break through the basal lamina and infiltrate into the underlying dermis. A summary of the epithelial tumors is found in Table 6-16.

The largest number of skin tumors (26 squamous papillomas and one squamous carcinoma in 12 mice) occurred in the positive control (Group 24-Urethane with TPA promoter). The next largest tumor numbers were with test sample #6 with TPA promoter (Group 11: 12 squamous papillomas in 12 mice) and vehicle (2% Emulphor) with TPA promoter (Group 22: 11 squamous papillomas in 11 mice).

All three test samples, when given with the TPA promoter, showed some degree of skin tumor development. Sample #6 is mentioned above. Samples #4 and #5 with TPA promoter (Group 7 and 9) each had four (in three mice) and four (in four mice) squamous papillomas, respectively.

The control group treated with 2% Emulphor and no promoter (Group 23) and all test samples without promoter (Groups 8, 10, 12) had no skin lesions.

Neoplasms in Organ Systems Other than Skin-- Malignant lymphomas were found in one Group 7 mouse (Sample #4 with promoter) and two Group 9 mice (Sample #5 with promoter). Alveolar/bronchiolar adenomas were found in one mouse each of Group 8 (Sample #4 without promoter), Group 11 (Sample #6 with promoter) and Group 22 (2% Emulphor with TPA promoter). These tumors do not appear to be compound related.

Table 6-16 Histopathology Findings: Skin Neoplasms and Lesions

Group	#Mice In Study	#Mice Sub. for Histo.	#Mice With Sq. Cell Carcinoma	#Mice With Sq. Cell Papilloma	#Mice With Epithelial Hyperplasia	#Mice With Skin Ulcer
7	40	17	0	3[a]	5	4
8	20	9	0	0	0	0
9	40	20	0	4	4	4
10	20	1	0	0	0	0
11	40	28	0	12	4	2
12	20	8	0	0	0	0
22	40	18	0	11	3	3
23	40	13	0	0	0	0
24	20	16	1[b]	12[c]	2	0

a - four sq. cell papillomas in 3 mice.

b - This animal also had a sq. cell papilloma and is included under the sq. cell papilloma heading.

c - 26 sq. cell papillomas in 12 mice.

Group 24 (Urethane with promoter) had five mice with alveolar/bronchiolar adenomas and an additional mouse with an alveolar-bronchiolar carcinoma. These tumors are probably related to urethane administration.

Non-neoplastic Lung Lesions-- Of 79 lungs examined histologically, 46 (58%) exhibited some degree of chronic interstitial inflammation. The lesion tended to be similar among the various animals and groups although varying occasionally in severity and specific cellular composition. The typical lesion tended to have a vascular orientation and was usually patchy in distribution. Admixed lymphocytes and macrophages infiltrated perivascular spaces and interalveolar septa. Some lesions took on a decidedly granulomatous appearance while others contained scattered foci of necrosis and neutrophilic infiltrates.

An apparent increase in incidence and severity of congestion/hemorrhage of the lungs was noted at necropsy by the Principal Investigator. This may relate to increased susceptibility of the stressed lungs to the euthanasia method (CO_2).

An infectious agent was suspected as the etiology of the lung inflammation due to the morphology of the lesions and the apparent random distribution throughout the test groups. No etiological agent, however, could be detected by regular stain (H&E) or by special stains for bacteria (B&H), mycobacteria/acid fast organisms (AF), or fungus/protozoa (GMS). Specific etiology of the lung lesions remains unclear at this time. Table 6-17 lists the non-neoplastic lung lesions by treatment group.

Miscellaneous Lesions-- Of thirty-one livers submitted for histopathology, 15 had varying degrees of coagulative necrosis usually with associated inflammation. The necrosis may be due to inherent local ischemia, however some form of association with the lung lesion cannot be totally ruled out.

Remaining lesions were identified and tabulated. A few were related to the presence of open skin lesions (extramedullary hematopoiesis or myeloid hyperplasia in liver and spleen; reactive lymph nodes). None appeared to be directly compound related or of other significance to warrant further comment. Except for the induction of lung adenomas in the group treated with urethane (a result expected from previous experimentation), the formation of other non-neoplastic or neoplastic lesions was not observed to result from treatment with test articles alone or in combination with TPA. Skin ulceration associated with inflammation and hyperplasia were observed in various groups as expected and found to be caused by the administration of TPA. Non-neoplastic lung lesions were observed at a higher than expected rate in all groups.

Overall results of this study - including sample toxicity findings, gross skin tumor data, and histopathology findings - indicate that samples 4-5L-P92, 5-5L-P94, and 6-5L-P96 are not acutely toxic, are not complete carcinogens for tumor formation, and are not initiators of TPA promoted tumors of the skin or organ systems, when tested in the Sencar bioassay.

Table 6-17 Histopathology Findings: Non-Neoplastic Lung Pathology

	Group								
	7	8	9	10	11	12	22	23	24
Mice sub. for histology	17	9	20	1	28	8	18	13	16
Lungs examined	10	5	9	1	16	7	10	12	9
Inflamm. interstitial, chronic	3	2	5	1	12	3	4	9	7
Lymph. infiltrate, perivascular	1	0	2	0	1	1	1	0	0
Congestion	10	5	9	1	16	7	9	12	5
Hemorrhage	2	3	5	0	2	5	4	2	3

6.3.3 MAI Assay Report 1840.04 (Samples 7-5L-P98, 8-5L-P30, 9-5L-P38)

Sample Toxicity--

Of 280 animals started on test, 17 animals died or were sacrificed in moribund condition prior to the sceduled terminal sacrifice at 30 experimental weeks. Eight animals were lost during the two week initiation phase of which seven showed signs of intubation accidents (punctured esophagus or trachea, hemorrhagic lungs, or hemorrhage and inflammation of the esophagus), and one showed a pitted liver and lymphatic enlargement. The single early death not associated with overt signs of intubation trauma occurred in an ainmal receiving test article #8. Deaths and moribund sacrifices occurring after the initiation period were limited to nine animals. Four post-initiation losses (two animals in group 14 and one animal each in groups 17 and 26) were associated with malignant lymphoma. The remaining five post-initiation losses were associated with TPA induced skin lesions (three in Group 17), lung hemorrhage and congestion (one in Group 25), and lymphopathy (one in Group 25). Administration of the test compound per se did not appear to result in significant toxicity (see Data Worksheet Files, MAI Assay Report 1840.04.

When compared to vehicle control animals (Group 26), a number of experimental groups showed statistically significant differences in their mean body weights at various times during the study when evaluated by a two-tailed *t*-test (alpha = 0.05). During the initiation phase (at one experimental week) both Groups 13 (compound #7) and 17 (compound #9) were significantly lighter than controls, but weight decreases were not consistently associated with those test articles since groups 14 and 18 receiving the same compounds did not show the same effect. During the post-initiation phase (3 to 30 experimental weeks) the following statistically significant decreases in body weight compared to controls were found. In Group 13 (compound #7 plus TPA) decreased weights were noted at 4, 5, 6, 10, 18, 22 and 26 experimental weeks. Group 14 (compound #7 plus acetone) was noted to have decreased body weights only at 22 experimental weeks. For Group 15 (compound #8 plus TPA) decreases were noted at 4, 5, 22, and 26 weeks, while Group 16 (compound #8 plus acetone) showed no differences at any time. For Group 17 (compound #9 plus TPA) decreases were noted at 22, 26, and 30 weeks, while Group 18 (compund #9 plus acetone) showed no differences at any time. Group 25 (vehicle plus TPA) showed significantly lighter weights at week 26 only. This pattern is puzzling and decreased body weights could not convincingly be attributed to the test articles or TPA per se. These findings and the survival results described above indicated that the test articles administered as described did not cause acute or delayed toxicities resulting in death or seriously altered body weights.

Gross Skin Tumor Data--

The gross scoring of skin tumors conducted during this assay proved to be quite accurate both in terms of the ability to detect a tumor and to classify them as papillomas or carcinomas. Histopathologic evaluations revealed three cases where skin carcinomas were not scored grossly. In two cases, they were masked by the ulcerative effects of TPA administration, and in the other, a lesion scored grossly as a papilloma was a skin carcinoma. Gross carcinomas were not detected during the 30 week course of the study and microscopic skin carcinomas were observed on only three animals in the study (one tumor on each, animals received test article #9 or urethane with TPA promotion). The extremely small number of detected carcinomas and their late detection in the assay eliminated the use of skin carcinoma formation as a parameter for evaluating the initiating potential of the test articles. The latency, incidence and multiplicity of cumulative skin papillomas (tumors persistent for three or more weekly tumor counts) were used as parameters for test evaluation.

The weekly count of actual skin tumors and cumulative skin tumors were recorded on the Lotus 1-2-3 worksheet files (see MAI Assay Report 1840.04) for each experimental group as were the percent incidence of animals with actual tumors or cumulative tumors, the total number of cumulative tumors, and the number of actual or cumulative tumors per surviving animal or animal promoted, respectively.

Table 6-18 summarizes the results of the assay based on the designated data parameters.

All groups receiving test article initiation with acetone promotion showed no skin tumor formation, and the test articles as administered did not act as complete carcinogens for skin tumor formation. The control group receiving initiator and promoter vehicles showed no skin tumor formation.

The positive control group receiving urethane initiation and TPA promotion showed a 65% incidence of animals with one or more cumulative papillomas by 30 experimental weeks and reached a 50% incidence at 18 experimental weeks. A total of 26 cumulative tumors were observed on 13 animals during the assay (1.3 cumulative tumors per effective animal or 2.0 cumulative tumors per cumulative tumor bearing animal). The first cumulative skin tumor was observed at 13 experimental weeks and the total number reached a maximum at 30 weeks. A total of 70% of the positive control animals showed skin tumors during the course of the study although some were not persistent cumulative tumors.

The control group receiving initiator vehicle and TPA promotion showed the formation of skin tumors, but the response compared to the positive control receiving urethane initiation and TPA promotion was considerably weaker in terms of the following parameters. The percent incidence of effective animals (those alive at the time of the first observation of a cumulative skin tumor in any experimental group) having one or more cumulative skin tumors by the 30th experimental week was 65% and 36% for the positive and TPA only controls, respectively. The number of cumulative skin tumors per effective animal at 30 experimental weeks was 1.3 and 0.38 in the positive and TPA only control, respectively. By the 30th experimental week the number of cumulative skin tumors per cumulative skin tumor bearing animal was 2.0 in the positive control and 1.1 in the

Table 6-18 MAI Assay Report 1840.04 Cumulative Skin Tumor Data Summary

Parameter	Group and Treatment (Sample:Promoter)[a] 13 7:T	 14 7:A	 15 8:T	 16 8:A	 17 9:T	 18 9:A	 25 Ve:T	 26 Ve:A	 27 Ur:T
# of animals started on test	40	20	40	20	40	20	40	40	20
# of animals reaching the 1st Promotion	39	19	38	18	40	20	39	39	20
Effective # of animals (number alive at the time of the 1st cumulative skin tumor observed in grp 27 at 13 weeks)	39	19	38	18	40	20	39	39	20
# of animals surviving til term. sac. at 30 experimental weeks	39	17	38	18	36	20	37	38	20
# of cumulative skin tumors through 30 experimental weeks	7	0	6	0	5	0	15	0	26
# of animals with at least 1 cum. skin tumor through 30 experimental weeks	5	0	6	0	5	0	14	0	13
% effective animals with cum. skin tumors through 30 experimental weeks	13%	0%	16%	0%	13%	0%	36%	0%	65%
Cumulative skin tumors per effective animal	0.18	0	0.16	0	0.13	0	0.38	0	1.3
Cumulative skin tumors per cumulative skin tumor bearing animal	1.4	NA	1.0	NA	1.0	NA	1.1	NA	2.0
Time of 1st observed cumulative skin tumor (in experimental weeks)	17	NA	18	NA	16	NA	15	NA	13
Time of maximum cumulative skin tumor count (in experimental weeks)	22	NA	27	NA	30	NA	29	NA	30
Cumulative % animals promoted which showed skin tumors regardless if persistent for 3 weeks	21%	0%	21%	0%	20%	0%	38%	0%	70%

[a] Sample:Promoter Key: A = Acetone, T = TPA, Ve = Vehicle, Ur = Urethane
NA = Not applicable

TPA control. Both groups showed their first cumulative skin tumor between 13-15 experimental weeks and reached a maximum cumulative skin tumor count between 29-30 experimental weeks. The cumulative percent of animals started on the promotion phase of the study which showed skin tumors (including cumulative tumors and those not persisting for three consecutive weekly counts) was 70% and 38% in the positive and TPA controls, respectively.

The ability of Sencar mice to form skin tumors following TPA promotion in the absence of a known chemical initiator has been reproducibly demonstrated by several investigators. However, in this study the 36 percent incidence of effective animals in the TPA control group showing one or more cumulative skin tumors by 30 experimental weeks was high. The two previous Sencar studies, MAI Assay Reports 1840.01 and 1840.03, demonstrated values of 9% and 25%, respectively.

The assay itself was considered valid since the incidence of cumulative skin tumor bearing animals in the positive control was statistically greater than the vehicle control when evaluated by the Chi-square test (0.05 level of significance). The fact that the TPA control group was relatively high for this parameter resulted in a demonstration of a positive response in the positive control at a higher level of significance (between 0.05 and 0.10).

The unusually high incidence of cumulative skin tumor bearing animals encountered in the TPA control through the 30-week experimental period did not seem to occur in the test groups receiving the test articles as initiators followed by TPA promotion. The latter groups demonstrated incidences comparable to the two previous studies (1840.01 and 1840.03), where test article initiation plus TPA promotion did not result in statistically significant increases in skin tumor formation compared to TPA controls.

In this study, initiation by the test articles followed by promotion with TPA yielded tumor incidences in animals (cumulative skin tumors by 30 experimental weeks) that were not significantly greater than the TPA only control. When analyzed by the Chi-square test (0.05 level of significance), there was no significant difference between the group receiving test article #8 and TPA compared to the TPA control group. The same statistical test revealed a significantly lower value for tumor incidence in the groups receiving test articles #7 or #9 and TPA promotion when compared to the TPA control group. Certainly, no statistically significant increases in tumor formation were observed when the test articles were used as potential initiators followed by TPA, and the decreases in tumor formation compared to the incidence found with TPA only were not statistically significant at an 0.025 level of significance. In the groups treated with the test articles and TPA, the number of cumulative skin tumors per effective animal was less than the TPA only control, but the number of cumulative tumors per cumulative tumor bearing animal was similar for all test article plus promotion groups and the promotion only control group (see Table 6-18). Time until a 50% incidence of cumulative skin tumor bearing animals was not a valid parameter for comparison of the test article plus promoter groups to the positive control, since the test article groups never attained higher than a 16% incidence. Although all of the test articles followed by TPA promotion resulted in a slightly longer latency period until first cumulative tumor appearance compared to the TPA only control, this effect was not striking and all test article groups formed their first cumulative skin tumors within three weeks of the TPA control. Another index based on latency is the average latency period for the appearance of all cumulative tumors on all tumor bearing animals within a group. Such an analysis indicated a mean latency (weeks) ± standard deviation for the appearance of all cumulative tumors of 19 ± 1.9, 22 ± 4.2, 21 ± 5.9, and 23 ± 4.2 for test articles #7, #8, or #9 plus TPA and the application of TPA only, respectively. There did not appear to be a significantly altered latency compared to the TPA only control.

Histopathology Findings--

The results of the histopathological evaluation are presented in detail within the PAI Pathology Report (MAI Assay Report 1840.04 Appendix A) which contains the following data:

1) all diagnosed tissue changes together with the number of organs examined, summarized by group in a Project Summary Table for non-neoplastic and neoplastic lesions

2) neoplasms alone, summarized for the entire study in Summary Tumor Incidence

3) neoplasms alone, summarized by group in a Tumor Incidence Table

4) a table of Tabulated Animal Data, correlating diagnoses by organ and by individual animal within each dosage group.

5) Individual Animal Data which includes microscopic observations by individual animal as well as gross to microscopic correlation where appropriate

After review of the detailed data tables, the histopathology results were summarized by PAI as follows:

Skin Neoplasms and Lesions-- Epithelial cell tumors occurred in mice from five of the nine groups and in 43 of the 233 mice from which tissues were examined. Squamous papillomas were diagnosed in 42 of these mice. One additional mouse had a squamous cell carcinoma (Group 17- Sample #9 with promoter) while two mice with squamous papillomas also had squamous cell carcinomas (both in Group 27-urethane with promoter). The delineation between the benign papilloma and the malignant carcinoma was based principally on two criteria: (1) the degree of atypia of the neoplastic cells and; (2) the tendency of the basilar layer of epidermal cells to break through the basal laminar and infiltrate into the underlying dermis. A summary of the number of epithelial tumors and lesions is given in Table 6-19.

Table 6-19 Histopathology Findings: Skin Neoplasms and Lesions

Group	#Mice In Study	#Mice Sub. for Histo.	#Mice With Sq. Cell Carcinoma	#Mice With Sq. Cell Papilloma	#Mice With Epithelial Hyperplasia	#Mice With Skin Ulcer
13	40	23	0	5	6	5
14	20	8	0	0	0	0
15	40	31	0	5	9	5
16	20	17	0	0	0	0
17	40	38	1	5	5	5
18	20	20	0	0	0	0
25	40	40	0	14[a]	4	1
26	40	37	0	0	0	0
27	20	19	2[b]	13[c]	4	2

a - 15 sq. cell papillomas in 14 mice.

b - These two animals also had sq. cell papillomas and are also included under sq. cell papilloma heading.

c - 22 sq. cell papillomas in 13 mice.

The largest number of skin tumors (22 squamous papillomas and two squamous carcinoma in 13 mice) occurred in the positive control (Group 27-Urethane with promoter). The next largest group was vehicle (2% Emulphor) with promoter (Group 25: 15 squamous papillomas in 14 mice). One test sample (Group 17- Sample #9 with promoter) had one squamous cell carcinoma and five squamous papillomas. The remaining two test samples with promoter had five squamous papillomas each.

The vehicle control group (2% Emulphor with no promoter; Group 26) and all test samples without promoter (Groups 14, 16, 18) had no skin tumors.

Neoplasms in Organ Systems Other Than Skin-- Malignant lymphomas were found in two Group 14 mice (Sample #7 without promoter) and in one mouse each in Groups 15 (Sample #8 with TPA promoter), 17 (Sample #9 with TPA promoter), and 26 (2% Emulphor without promoter). Uterine deciduomas were found in one mouse each in Groups 13 (Sample 7 with promoter), 16 (Sample 8 without promoter), and 26 (2% Emulphor with no promoter). These tumors do not appear to be compound related.

Single alveolar/bronchiolar adenomas were found in four mice of Group 15 (Sample 8 with promoter), 3 mice in Group 16 (Sample 8 without promoter), 3 in Group 25 (2% Emulphor and promoter), and 2 in Group 26 (2% Emulphor with no promoter). Although there appears to be a possible increased incidence of these tumors in association with Sample #8, this is believed to be secondary to sampling size and not of significance.

Group 27 (Urethane with TPA promoter) had 8 mice with a total of 17 alveolar/bronchiolar adenomas. These tumors are probably related to urethane administration.

Non-neoplastic Lung Lesions-- Of 179 lungs examined histologically, 120 (67%) exhibited some degree of interstitial inflammation (interstitial inflammation and chronic interstitial inflammation have been combined). The lesion tended to be similar among the various animals and groups although varying in severity (usually minimal to mild). The typical lesion tended to be patchy in distribution and chronic in nature consisting predominately of lymphocytes and macrophages. Some lesions appeared as granulomatous or foreign-body type reactions. A few of the more severe lesions were more subchronic in cellular composition with admixed neutrophils present. Overall, the lesions tended to be much less severe and more chronic than those noted in a previous study from this colony (MAI Assay Report 1840.03).

An apparent increase in incidence and severity of congestion/ hemorrhage of the lungs was noted at necropsy by the Principal Investigator. This may relate to increased susceptibility of the stressed lungs to the euthanasia method (CO_2). Specific etiology of the lung lesions remains unclear at this time. Table 6-20 lists the lung lesions by treatment group.

Table 6-20 Histopathology Findings: Non-Neoplastic Lung Pathology

	Group								
	13	14	15	16	17	18	25	26	27
Mice sub. for histo	23	8	31	17	38	20	40	37	19
Lungs examined	7	5	20	16	28	19	32	35	17
Inflamm. Interstitial[a]	2	3	16	12	21	11	21	24	10
Lymph. Infiltrate	2	1	6	3	1	1	0	0	2
Congestion	5	4	19	16	27	19	32	34	16
Hemorrhage	6	2	15	10	16	7	17	25	10
Alv./bronch. adenoma	0	0	4	3	0	0	3	2	8[b]

[a] Inflammation, interstitial, chronic and inflammation, interstitial (not otherwise specified) are combined.

[b] Total of 17 alveolar/bronchiolar (alv./bronch.) adenomas in 8 mice.

Miscellaneous Lesions-- Of thirty-one livers submitted for histopathology, 21 had varying degrees of coagulative necrosis usually with associated inflammation. The necrosis may be due to inherent local ischemia.

Remaining lesions were identified and tabulated. A few were related to the presence of open skin lesions (extramedullary hematopoiesis or myeloid hyperplasia in liver and spleen; reactive lymph nodes). None appeared to be directly compound related or of other significance to warrant further comment.

Except for the induction of lung adenomas in the group treated with urethane (a result expected from previous experimentation), the formation of other non-neoplastic or neoplastic lesions was not observed to result from treatment with test articles alone or in combination with TPA. Skin ulceration associated with inflammation and hyperplasia were observed in various groups as expected and found to be caused by the administration of TPA. Non-neoplastic lung lesions were observed at a higher than expected rate in all groups. Alveolar/bronchiolar adenomas were found in mice dosed with sample #8 but the incidence was believed to be secondary to sampling size and not of significance.

Overall results of this study including sample toxicity findings, gross skin tumor data, and histopathology findings indicate that samples 7-5L-P98, 8-5L-P30, and 9-5L-P38 are not acutely toxic, are not complete carcinogens for tumor formation, and are not initiators of TPA promoted tumors of the skin or organ systems, when tested in the Sencar bioassay.

6.3.4 Submission of Study Materials

All residual quantities of samples, residual paraffin-embedded blocks of gross lesions, formalin-preserved necropsy tissues, photographic slides of skin tumors, Lotus 1-2-3 computer disc files, and stained pathology slides were submitted to the sponsor at or prior to submission of this Final Report.

6.4 RAT LIVER FOCUS ASSAYS

6.4.1 Nine Sample Series (MAI Assay Report 1840.02)

Histochemical Evaluations--

For reporting of results, treatment groups dosed with samples or controls were designated as follows:

Treatment Group	Dosed With
Group 1	Sample 1-5L-P86
Group 2	Sample 2-5L-P88
Group 3	Sample 3-5L-P90
Group 4	Sample 4-5L-P92
Group 5	Sample 5-5L-P94
Group 6	Sample 6-5L-P96
Group 7	Sample 7-5L-P98
Group 8	Sample 8-5L-P30
Group 9	Sample 9-5L-P38
Group 10	Vehicle Control, 2% Emulphor
Group 11	Positive Control, DEN

PAI observed no foci in the histochemical evaluation of slides prepared from Groups 2,3,4,6,8,9, or 10. One focus was seen in one out of the 9 animals evaluated in Group 1. Only 2 foci were seen in Group 5 which had 9 animals in the group. Both of these foci were seen in the same animal. One focus was seen in one out of the 9 animals evaluated in Group 7. A total of 41 foci were seen in the eight animals evaluated in Group 11, the positive control group. At least one focus was seen in each of the eight animals. However, one animal had only 1.78 square centimeters of liver examined and therefore, it was not used in the statistical evaluation. This left seven animals in Group 11 with greater than 2.00 square centimeters of liver evaluated. Out of these seven animals, all had one or more foci with a total of 36 foci for the group.

Mathematical treatments of the raw data, corresponding to the five required data parameters, were summarized for each of the eleven treatment groups and are presented in Table 6-21, along with PAI's statistical analysis of the validity of these parameters.

PAI determined that the individual GGTase focus area, the number of GGTase positive foci/cm^2 of liver evaluated, the number of GGTase positive foci/cm^3 of liver evaluated and percentage of liver area evaluated exhibiting GGTase positive foci were valid tests as there was a significant difference between the positive control and the vehicle control groups (Groups 11 and 10 respectively) for each of these parameters. When each of the remaining groups was compared to the vehicle control for all the above parameters, it was determined that there was no significant difference between any of the groups and the vehicle control. MAI disagrees with PAI's determination of individual focus area as a valid parameter, determining that the mean GGT+ focus area for the vehicle control was not significantly different from the positive control group since no vehicle control foci were scored to be subsequently

Table 6-21 Evaluation of Nine Sample Series in Rat Liver Focus Assay: Summary of Histochemical Findings and Statistical Analysis

Data Parameter	Group (Mean) 1	2	3	4	5	6	7	8	9	10	11
Individual GGTase⁺ Focus Area ($cm^2 \times 10^{-4}$) [1]	4.52	0.00	0.00	0.00	3.83	0.00	38.48	0.00	0.00	0.00	18.85
GGTase⁺ Foci/cm^2 Liver Evaluated [1]	0.04	0.00	0.00	0.00	0.06	0.00	0.04	0.00	0.00	0.00	1.70
GGTase⁺ Foci/cm^3 Liver Evaluated [1]	1.05	0.00	0.00	0.00	1.71	0.00	0.34	0.00	0.00	0.00	34.40
% of Liver Evaluated Exhibiting GGTase⁺ Zonal Induction [2]	8.64	6.17	6.52	11.11	4.71	8.78	8.88	5.80	3.45	2.62	3.47
% of Liver Evaluated Exhibiting GGTase⁺ Foci [1]	0.002	0.00	0.00	0.00	0.002	0.00	0.01	0.00	0.00	0.00	0.29

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[1] Gps 10 vs 11	Gp 11 is significantly different from Gp 10
Gps 1 thru 9 vs Gp 10	Gps 1 thru 9 are not significantly different from Gp 10
[2] Gps 1,4,6 and 7 vs Gp 10	Gps 1,4,6 and 7 are significantly different from Gp 10
Gps 2,3,5,8,9 and 11 vs Gp 10	Gps 2,3,5,8,9 and 11 are not significantly different from Gp 10
Gps 1 thru 9 vs Gp 11	Gps 1,3,4, and 7 are significantly different from Gp 11
	Gps 2,5,6,8, and 9 are not significantly different from Gp 11

Table 6-22 Survival Summary for the Nine Sample Series

Group	Test Article Dosed	# Hepatectomized	# Alive at 24 hrs After pHx and Dosed	# Sacrificed due to Regurgitation of Test Item	# Lost During Resuturing of Surgical Incisions (Anesthesia deaths) Not Toxicity	Percent (#) Dead After Dosing and Assumed Due to Toxicity	# Dead or Sacrificed After 1 wk on Test	# Surviving at 10 wk Terminal Sacrifice
1	1-5L-P86	10	10	0	0	10(1***)	0	9
2	2-5L-P88	10	10	0	0	0(0)	0	10
3	3-5L-P90	10	10	0	0	0(0)	0	10
4	4-5L-P92	11	11	0	0	9(1*)	0	10
5	5-5L-P94	13	13	0	0	31(2*) (2**)	0	9
6	6-5L-P96	14	14	0	0	64(4*) (5**)	0	5
7	7-5L-P98	12	12	0	0	25(2*) (1**)	0	9
8	8-5L-P30	10	10	0	0	0(0)	0	10
9	9-5L-P38	10	10	0	0	0(0)	0	10
10	2% Emulphor	10	10	0	1	0(0)	0	9
11	50mg DEN/Kg	11	11	1	0	0(0)	2 +	8

*Dead immediately after test item dosing
**Dead within 48 hours of test item dosing
***Dead within 1 week of test item dosing
+Deaths or sacrifices associated with complication from hepatic surgery

characterized by a mean size, SEM, and sample size. Consequently, comparison of the sample groups and the vehicle control were also invalid.

Both PAI and MAI question the use and significance of the percentage of liver evaluated exhibiting GGTase positive zonal induction (ZI) as a parameter to measure initiation by the nine test substances, since there was no significant difference between the positive and vehicle control groups for ZI. However, there was a significant difference between the vehicle control (Group 10) and some of the test groups (Groups 1, 4, 6 and 7) for ZI. There was also a significant difference between the positive control (Group 11) and some ofthe test groups (Groups 1, 3, 4, 7). The reason for these findings is unclear and can not be determined from the experiment as designed. It may be that one dose of the test compound either alone or in combination with one or more of the following-phenobarbital, Emulphor, or partial hepatectomy-may have in some way influenced the formation of the GGTase positive zonal induction. The above responses suggest that the ability of the compounds in this study to initiate GGTase positive foci does not correlate with their ability to initiate production of GGTase positive zonal induction.

Overall, taking into account both the raw data and the statistical analysis of the calculated data parameters, PAI and MAI concluded that none of the nine samples, under the specific test conditions, were significant initiators of GGTase positive foci.

Sample Toxicity--

Although not designed to evaluate the toxicity of the test articles, this study did reveal preliminary data relating to the relative toxicity of the concentrated water samples. Table 6-22 summarizes the survival of those animals started on the study through the ten week duration of the test and categorized the known or suspected cause of death in each group. Since gross and microscopic pathology evaluations were not required by the study protocol, a definitive cause of death cannot be presented. The percent of deaths within 1 week of intragastric administrations of the test articles indicated possible acute toxicities for compounds 6, 5, 7, 4, and 1 (in that relative order of decreasing toxicities). It is unlikely that deaths were caused by other experimental factors such as gavaging accidents, surgery, etc. or the distribution of losses would have been more random and approximately equivalent in all groups, since all were similarly treated with the exception of the compounds that were administered 24 hours after surgery.

6.4.2 Five Sample Series (MAI Report 1840.05)

Histochemical Evaluations--

For reporting of results, treatment groups dosed with samples or controls were designated as follows:

Treatment Group	Dosed With
Group 1	Sample 1-9/84
Group 2	Sample 2-9/84
Group 3	Sample 3-9/84
Group 4	Sample 4-9/84
Group 5	Sample 5-9/84
Group 6	Vehicle Control, 2% Emulphor
Group 7	Positive Control, DEN

PAI observed foci in the histochemical evaluation of slides prepared from all seven groups. A total of seven foci were seen in Group 1, in three of nine animals. A total of seven foci were counted in Group 2, with six out of the 10 animals in the group positive for foci. A total of nine foci were seen in Group 3, in five of 10 animals. A total of two foci were observed in Group 4, both occuring in one animal. A total of five foci were counted in Group 5, in four of 10 animals. A total of four foci were seen in Group 6, in three of nine animals. A total of 142 foci occurred in Group 7, with all nine animals in the group having one or more foci.

Mathematical treatments of the raw data, corresponding to the five required data parameters, were summarized for each of the seven treatment groups and are presented in Table 6-23, along with PAI's statistical analysis of the validity of these parameters.

PAI determined that the individual GGTase foci area, the number of GGTase positive foci/cm^2 of liver evaluated, the number of GGTase positive foci/cm^3 of liver evaluated and percentage of liver area evaluated exhibiting GGTase positive foci were valid tests as there was a significant difference between the positive control and the vehicle control groups (Groups 7 and 6 respectively) for each of these parameters. When each of the remaining groups (Groups 1 thru 5) were compared to the vehicle control for all the above parameters, it was determined that there was no significant difference between any of the groups and the vehicle control.

Both PAI and MAI again question the use and the significance of the percentage of liver evaluated exhibiting GGTase positive zonal induction (ZI) as a parameter to measure the initiating ability of the five test substances, since there was no significant difference between the positive and vehicle control groups for ZI. Nor did any of the animals in the test groups have a significant increase in the amount of GGTase positive zonal induction. This is in contrast to the Nine Sample study (Section 6.4.1) in which some of the test groups did have a significant increase in the amount of GGTase positive zonal induction when compared to vehicle or positive controls. The results of this study suggest that the ability of a compound to initiate GGTase positive foci does not correlate with its ability to initiate production of GGTase positive zonal induction. This is supported by the findings in Group 7 which had a significantly greater number of GGTase positive foci than any of the other groups, but did not have a significantly greater amount of GGTase positive zonal induction.

Table 6-23 Evaluation of Five Sample Series in Rat Liver Focus Assay: Summary of Histochemical Findings and Statistical Analysis

Data Parameter	1	2	3	4	5	6	7
Individual GGTase$^+$ Focus Area ($cm^2 \times 10^{-4}$) [1]	0.87	1.27	1.90	0.96	2.56	1.23	1.82
GGTase$^+$ Foci/cm^2 Liver Evaluated [1]	0.21	0.22	0.27	0.08	0.15	0.13	4.37
GGTase$^+$ Foci/cm^3 Liver Evaluated [1]	15.02	12.11	13.98	4.63	6.19	6.39	211.33
% of Liver Evaluated Exhibiting GGTase$^+$ Zonal Induction [2]	3.30	3.97	3.41	5.50	4.06	1.68	3.39
% of Liver Evaluated Exhibiting GGTase$^+$ Foci [1]	0.002	0.003	0.005	0.001	0.004	0.001	0.079

[1] Gp 6 vs Gp 7	Gp 7 is significantly different from Gp 6
Gps 1 thru 5 vs Gp 6	Gps 1 thru 5 are not significantly different from Gp 6
[2] Gp 6 vs Gp 7	Gp 6 is not significantly different from Gp 7
Gps 1 thru 5 vs Gp 6	Gps 1 thru 5 are not significantly different from Gp 6

Table 6-24 Survival Summary for the Five Sample Series

Group	Test Article Dosed	# Hepatectomized	# Alive at 24 hrs After pHx and Dosed	# Sacrificed due to Regurgitation of Test Item	Sacrificed or Dead due to Surgical Complications	Percent(#) Dead After Dosing	# Dead or Sacrificed After 1 wk on Test	# at 10 wk Terminal Sacrifice
1	1-9/84	10	10	0	0	10(1**)	0	9
2	2-9/84	12	12	0	2	0(0)	0	10
3	3-9/84	13	13	1	2	0(0)	0	10
4	4-9/84	11	11	0	0	18(1*) (1***)	0	9
5	5-9/84	10	10	0	0	0(0)	0	10
6	2% Emulphor	10	10	0	1	0(0)	0	9
7	50 mg DEN/kg	11	11	0	1	10(1**)	0	9

* Dead immediately after test item dosing
** Dead within 24 hours of test item dosing
*** Dead within 72 hours of test item dosing

Overall, taking into account both the raw data and the statistical analysis of the calculated data parameters, PAI and MAI concluded that none of the five samples, under the specific test conditions, were significant initiators of GGTase positive foci.

Sample Toxicity--

The mortality of animals receiving intragastric doses of the test articles, test article vehicle, or the known initiator (DEN) are presented in Table 6-24 together with the cause of death when known. Excluding sacrifices required by regurgitation of the intragastrically delivered compounds and the sacrifice of animals with surgical complications (serious hernias resulting from the loss of sutures that could not be repaired), all other deaths were encountered within 72 hours of dosing. No gross or microscopic pathologic evaluations of dead animals were required by the protocol, unexplained deaths were limited to no more than two animals per group, and the pattern of death did not indicate a significantly increased incidence in any particular treatment group. These facts would make it difficult to ascribe any toxicity to the test compounds. The pattern of deaths could have been as easily explained by other general experimental factors such as undetected surgical complications or faulty intragastric dosings resulting in delivery of some of the compounds to the resipiratory tract by aspiration or direct instillations. Asphyxiation or damage to respiratory tract by the test compounds could have resulted in some of the deaths and not been due to toxicity through the gastric route.

6.4.3 Submission of Study Materials

All residual quantities of the samples, all residual frozen blocks, all stained liver sections, and all low-melting point paraffin blocks were submitted to the sponsor at or prior to submission of this Final Report.

6.5 Lung Adenoma Assays

6.5.1 Nine Sample Series (EHRT Study No. ETOX-84-1001-02)

Sample Toxicity--

Body weight changes during the study were not affected by the urethane (positive control) or the concentrated water samples (Tables 6-25 and 6-26). Mortality data also indicated no treatment-related effect (Tables 6-27 and 6-28); the overall mortality rate in this study was 5.8% (19 males and 30 females for a total of 49 dead out of 840 mice). In agreement with these findings, results of the detailed health status checks revealed no observable toxic effects.

Lung Adenoma Counts--

The incidence of lung adenomas was 100% for both the male and female positive control groups (Tables 6-29 and 6-30), with cumulative lung nodule indexes of 11.78 and 11.66 (Tables 6-31 and 6-32) for males and females, respectively. Animals in the untreated and vehicle control groups showed a much weaker response, with tumor incidences ranging from 5 to 10% and cumulative lung nodule indexes from 0.05 to 0.10.

The incidence of lung adenomas in animals treated with the nine coded samples varied from 0 to 30% among males and 0 to 28% among females while the average number of tumors per animal ranged from 0 to 0.35 in the males and 0 to 0.33 in the females. When these responses were compared to those observed in the untreated and vehicle control groups, there were no indications that any of these samples were initiators of lung adenomas.

Examination of the lungs, both grossly and microscopically, did not reveal the presence of any abnormalities, other than the nodules discussed above.

Histopathology Findings--

Results of the histopathological examination of selected tumor-bearing lungs are shown in Table 6-33. A total of 85 adenomas were confirmed histopathologically out of a total of 96 observed grossly, yielding an overall correlation of 89%.

The overall results of this study - including sample toxicity, gross adenoma counts, and histopathology findings - indicate that none of the nine test samples were acutely toxic and none were initiators of lung adenomas in the Strain A mouse under the specific test conditions employed.

Table 6-25 Changes In Body Weights (Males)

Animal Nos.	Sample No.	Concentration Level	Change (G)
351-370	----	Untreated Ctrl	8.08
371-390	2% Emulphor	Vehicle Ctrl	9.55
391-410	Urethane	Positive Ctrl	7.50
411-430	1-5L-P-86	1/2 X	9.79
431-450	2-5L-P-88	1/2 X	8.57
451-470	3-5L-P-90	1/2 X	7.83
471-490	4-5L-P-92	1/2 X	7.74
491-510	5-5L-P-94	1/2 X	10.22
511-530	6-5L-P-96	1/4 X	7.16
531-550	7-5L-P-98	1/2 X	8.35
551-570	8-5L-P-30	1/2 X	8.65
571-590	9-5L-P-38	1/2 X	8.09
591-610	1-5L-P-86	1 X	9.87
611-630	2-5L-P-88	1 X	9.15
631-650	3-5L-P-90	1 X	7.47
651-670	4-5L-P-92	1 X	7.26
671-690	5-5L-P-94	1 X	9.69
691-710	6-5L-P-96	1/2 X	8.42
711-730	7-5L-P-98	1 X	9.08
731-750	8-5L-P-30	1 X	8.70
751-770	9-5L-P-38	1 X	9.58

Table 6-26 Changes In Body Weights (Females)

Animal Nos.	Sample No.	Concentration Level	Change (G)
771-790	----	Untreated Ctrl	5.26
791-810	2% Emulphor	Vehicle Ctrl	4.51
811-830	Urethane	Positive Ctrl	5.27
831-850	1-5L-P-86	1/2 X	6.04
851-870	2-5L-P-88	1/2 X	4.72
871-890	3-5L-P-90	1/2 X	4.94
891-910	4-5L-P-92	1/2 X	5.49
911-930	5-5L-P-94	1/2 X	5.84
931-950	6-5L-P-96	1/4 X	6.03
951-970	7-5L-P-98	1/2 X	5.09
971-990	8-5L-P-30	1/2 X	4.78
991-1010	9-5L-P-38	1/2 X	3.84
1011-1030	1-5L-P-86	1 X	4.64
1031-1050	2-5L-P-88	1 X	5.51
1051-1070	3-5L-P-90	1 X	4.25
1071-1090	4-5L-P-92	1 X	5.27
1091-1110	5-5L-P-94	1 X	4.66
1111-1130	6-5L-P-96	1/2 X	5.05
1131-1150	7-5L-P-98	1 X	4.78
1151-1170	8-5L-P-30	1 X	3.41
1171-1190	9-5L-P-38	1 X	3.95

Table 6-27 Mortality Data (Males)

Animal Nos.	Sample No.	Concentration Level	Dead
351-370	----	Untreated Ctrl	1A
371-390	2% Emulphor	Vehicle Ctrl	1A,4B
391-410	Urethane	Positive Ctrl	0
411-430	1-5L-P-86	1/2 X	2A
431-450	2-5L-P-88	1/2 X	0
451-470	3-5L-P-90	1/2 X	0
471-490	4-5L-P-92	1/2 X	0
491-510	5-5L-P-94	1/2 X	0
511-530	6-5L-P-96	1/4 X	0
531-550	7-5L-P-98	1/2 X	0
551-570	8-5L-P-30	1/2 X	0
571-590	9-5L-P-38	1/2 X	1A
591-610	1-5L-P-86	1 X	3A
611-630	2-5L-P-88	1 X	1A
631-650	3-5L-P-90	1 X	1A,3C
651-670	4-5L-P-92	1 X	0
671-690	5-5L-P-94	1 X	0
691-710	6-5L-P-96	1/2 X	1A
711-730	7-5L-P-98	1 X	0
731-750	8-5L-P-30	1 X	1A
751-770	9-5L-P-38	1 X	0

A = died from gavage trauma
B = accidentally drown
C = died from unknown causes

Table 6-28 Mortality Data (Females)

Animal Nos.	Sample No.	Concentration Level	Dead
771-790	----	Untreated Ctrl	0
791-810	2% Emulphor	Vehicle Ctrl	0
811-830	Urethane	Positive Ctrl	1D
831-850	1-5L-P-86	1/2 X	0
851-870	2-5L-P-88	1/2 X	0
871-890	3-5L-P-90	1/2 X	0
891-910	4-5L-P-92	1/2 X	2A
911-930	5-5L-P-94	1/2 X	1A
931-950	6-5L-P-96	1/4 X	0
951-970	7-5L-P-98	1/2 X	0
971-990	8-5L-P-30	1/2 X	0
991-1010	9-5L-P-38	1/2 X	0
1011-1030	1-5L-P-86	1 X	2A
1031-1050	2-5L-P-88	1 X	2A,3D
1051-1070	3-5L-P-90	1 X	3A
1071-1090	4-5L-P-92	1 X	7D
1091-1110	5-5L-P-94	1 X	2A
1111-1130	6-5L-P-96	1/2 X	1A
1131-1150	7-5L-P-98	1 X	1D
1151-1170	8-5L-P-30	1 X	1D
1171-1190	9-5L-P-38	1 X	4D

A = died from gavage trauma

D = died of dehydration

Table 6-29 Percent Tumor-Bearing Animals (Males): Average of Two Counts

Animal Nos.	Sample No.	Concentration Level	% TBA
351-370	----	Untreated Ctrl	5.3
371-390	2 % Emulphor	Vehicle Ctrl	10.0
391-410	Urethane	Positive Ctrl	100.0
411-430	1-5L-P-86	1/2 X	25.0
431-450	2-5L-P-88	1/2 X	30.0
451-470	3-5L-P-90	1/2 X	15.0
471-490	4-5L-P-92	1/2 X	12.5
491-510	5-5L-P-94	1/2 X	5.0
511-530	6-5L-P-96	1/4 X	0.0
531-550	7-5L-P-98	1/2 X	10.0
551-570	8-5L-P-30	1/2 X	5.0
571-590	9-5L-P-38	1/2 X	0.0
591-610	1-5L-P-86	1 X	5.3
611-630	2-5L-P-88	1 X	5.0
631-650	3-5L-P-90	1 X	25.0
651-670	4-5L-P-92	1 X	5.0
671-690	5-5L-P-94	1 X	5.0
691-710	6-5L-P-96	1/2 X	5.3
711-730	7-5L-P 98	1 X	10.0
731-750	8-5L-P-30	1 X	7.9
751-770	9-5L-P-38	1 X	15.0

TBA = Tumor-Bearing Animals

Table 6-30 Percent Tumor-Bearing Animals (Females): Average of Two Counts

Animal Nos.	Sample No.	Concentration Level	% TBA
771-790	----	Untreated Ctrl	5.0
791-810	2% Emulphor	Vehicle Ctrl	5.0
811-830	Urethane	Positive Ctrl	100.0
831-850	1-5L-P-86	1/2 X	20.0
851-870	2-5L-P-88	1/2 X	5.0
871-890	3-5L-P-90	1/2 X	0.0
891-910	4-5L-P-92	1/2 X	11.1
911 930	5-5L-P-94	1/2 X	0.0
931-950	6-5L-P-96	1/4 X	0.0
951-970	7-5L P-98	1/2 X	5.0
971-990	8-5L-P-30	1/2 X	25.0
991 1010	9-5L-P-38	1/2 X	5.0
1011-1030	1-5L-P-86	1 X	16.7
1031-1050	2-5L-P-88	1 X	6.7
1051-1070	3-5L-P-90	1 X	11.8
1071-1090	4-5L-P-92	1 X	7.7
1091-1110	5-5L-P-94	1 X	5.3
1111-1130	6-5L-P-96	1/2 X	5.3
1131-1150	7-5L-P-98	1 X	10.5
1151-1170	8-5L-P-30	1 X	26.3
1171-1190	9-5L-P-38	1 X	28.0

TBA = Tumor-Bearing Animals

Table 6-31 Cumulative Nodules Per Animal (Males): Average of Two Counts

Animal Nos.	Sample No.	Concentration Level	Mean ± SD
351-370	----	Untreated Ctrl	0.05 ± 0.22
371-390	2% Emulphor	Vehicle Ctrl	0.10 ± 0.30
391-410	Urethane	Positive Ctrl	11.78 ± 3.38
411-430	1-5L-P-86	1/2 X	0.25 ± 0.44
431-450	2-5L-P-88	1/2 X	0.35 ± 0.57
451-470	3-5L-P-90	1/2 X	0.18 ± 0.44
471-490	4-5L-P-92	1/2 X	0.18 ± 0.50
491-510	5-5L-P-94	1/2 X	0.05 ± 0.22
511-530	6-5L-P-96	1/4 X	0.00 ± 0.00
531-550	7-5L-P-98	1/2 X	0.13 ± 0.39
551-570	8-5L-P-30	1/2 X	0.05 ± 0.22
571-590	9-5L-P-38	1/2 X	0.00 ± 0.00
591-610	1-5L-P-86	1 X	0.06 ± 0.24
611-630	2-5L-P-88	1 X	0.05 ± 0.22
631-650	3-5L-P-90	1 X	0.31 ± 0.58
651-670	4-5L-P-92	1 X	0.05 ± 0.22
671-690	5-5L-P-94	1 X	0.05 ± 0.22
691-710	6-5L-P-96	1/2 X	0.05 ± 0.22
711-730	7-5L-P-98	1 X	0.15 ± 0.45
731-750	8-5L-P-30	1 X	0.08 ± 0.27
751-770	9-5L-P-38	1 X	0.15 ± 0.36

SD = Standard Deviation

Table 6-32 Cumulative Nodules Per Animal (Females): Average of Two Counts

Animal Nos.	Sample No.	Concentration Level	Mean ± SD
771-790	----	Untreated Ctrl	0.05 ± 0.22
791-810	2% Emulphor	Vehicle Ctrl	0.05 ± 0.22
811-830	Urethane	Positive Ctrl	11.66 ± 3.79
831-850	1-5L-P-86	1/2 X	0.20 ± 0.40
851-870	2-5L-P-88	1/2 X	0.05 ± 0.22
871-890	3-5L-P-90	1/2 X	0.00 ± 0.00
891-910	4-5L-P-92	1/2 X	0.11 ± 0.31
911-930	5-5L-P-94	1/2 X	0.00 ± 0.00
931-950	6-5L-P-96	1/4 X	0.00 ± 0.00
951-970	7-5L-P-98	1/2 X	0.05 ± 0.22
971-990	8-5L-P-30	1/2 X	0.33 ± 0.65
991-1010	9-5L-P-38	1/2 X	0.05 ± 0.22
1011-1030	1-5L-P-86	1 X	0.17 ± 0.37
1031-1050	2-5L-P-88	1 X	0.07 ± 0.25
1051-1070	3-5L-P-90	1 X	0.18 ± 0.53
1071-1090	4-5L-P-92	1 X	0.08 ± 0.27
1091-1110	5-5L-P-94	1 X	0.06 ± 0.23
1111-1130	6-5L-P-96	1/2 X	0.08 ± 0.34
1131-1150	7-5L-P-98	1 X	0.11 ± 0.31
1151-1170	8-5L-P-30	1 X	0.32 ± 0.57
1171-1190	9-5L-P-38	1 X	0.32 ± 0.52

SD = Standard Deviation

Table 6-33 Correlation of Gross Counts with Histopathology Findings

Animal No.	Observer/ Gross Counts 1	2	3[a]	Confirmed by Histopathology Slide Count	% Correlation[b]
355	1	1	1	0	0%
377	1	0	0	0	100%
393	10	11	10	10	100%
401	17	16	17	14	82%
413	1	1	1	1	100%
431	1	1	1	0	0%
451	1	2	2	1	50%
473	1	1	1	1	100%
502	1	1	1	1	100%
533	2	1	1	1	100%
553	1	1	1	1	100%
605	1	1	1	1	100%
606	0	0	2	2	100%
629	1	1	1	1	100%
636	1	1	1	0	0%
665	1	1	1	0	0%
671	1	1	1	1	100%
710	1	1	2	2	100%
711	2	1	2	2	100%
743	1	1	1	1	100%
761	1	1	1	1	100%
778	1	1	1	1	100%
798	1	1	2	2	100%
812	14	15	14	13	93%
828	13	13	12	12	100%
835	1	1	1	1	100%
865	1	1	1	0	0%
898	1	1	1	0	0%
957	1	1	1	1	100%
974	1	1	1	1	100%
992	1	1	1	1	100%
1012	1	1	1	1	100%
1047	1	1	1	1	100%
1060	1	3	2	3	100%
1080	1	1	1	1	100%
1110	1	1	1	1	100%
1125	2	1	2	2	100%
1132	1	1	1	1	100%
1151	2	2	2	2	100%
1176	1	1	1	1	100%
TOTALS	93	93	96	85	89%

[a] Reconciled count

[b] Correlation with histopathology was based upon the reconciled gross count

6.5.2 Five Sample Series (EHRT Study No. ETOX-84-1001-04)

Sample Toxicity--

Body weight changes during the study were not affected by treatment with urethane (positive control) or the concentrated water samples (Tables 6-34 and 6-35). Mortality data also indicated no treatment-related effect (Tables 6-36 and 6-37); the mortality rate in this study was 1.9% (5 males and 5 females for a total of 10 dead out of 520 mice). In agreement with these findings results of the detailed health status checks revealed no observable toxic effects.

Lung Adenoma Counts--

The incidence of lung adenomas was 30% for the male positive control group and 35% for the female group (Tables 6-38 and 6-39), with a cumulative lung nodule index of 0.40 for both males and females (Tables 6-40 and 6-41). These responses were much lower than those observed in the Nine Sample Study. Animals in the untreated and vehicle control groups yielded tumor incidences ranging from 5.6 to 21% and cumulative lung nodule indexes from 0.06 to 0.42.

The incidence of lung adenomas in animals treated with the five coded samples varied from 10 to 35% among males and 0 to 25% among females while the average number of tumors per animal ranged from 0.10 to 0.35 in the males and 0 to 0.30 in the females. When these responses were compared to those observed in the untreated and vehicle control groups, there were no indications that any of these samples were initiators of lung adenomas.

Examination of the lungs, both grossly and microscopically, did not reveal the presence of any abnormalities, other than the nodules discussed above.

Histopathology Findings--

Results of the histopathological examination of selected tumor-bearing lungs are shown in Table 6-42. A total of 8 adenomas were confirmed histopathologically out of a total of 9 observed grossly, yielding an overall correlation of 89%.

Overall results of the study - including sample toxicity, gross adenoma counts, and histopathology findings - indicate that none of the five test samples were acutely toxic and none were initiators of lung adenomas in the Strain A mouse under the specific test conditions employed.

6.5.3 Submission of Study Materials

All residual quantities of samples, photographs of tumor-bearing lungs, preserved lung tissues, and histopathology slides were submitted to the sponsor at or prior to submission of this Final Report.

Table 6-34 Changes in Body Weights (Males)

Animal Nos.	Sample No.	Concentration Level	Change (G)
1451-1470	----	Untreated Ctrl	7.23
1471-1490	2% Emulphor	Vehicle Ctrl	6.96
1491-1510	Urethane	Positive Ctrl	7.36
1511-1530	1-9/84	1/2 X	6.75
1531-1550	2-9/84	1/2 X	5.92
1551-1570	3-9/84	1/2 X	7.05
1571-1590	4-9/84	1/2 X	6.33
1591-1610	5-9/84	1/2 X	6.77
1611-1630	1-9/84	1 X	7.64
1631-1650	2-9/84	1 X	7.17
1651-1670	3-9/84	1 X	7.24
1671-1690	4-9/84	1 X	6.58
1691-1710	5-9/84	1 X	7.27

Table 6-35 Changes in Body Weights (Females)

Animal Nos.	Sample No.	Concentration Level	Change (G)
1711-1730	----	Untreated Ctrl	5.83
1731-1750	2% Emulphor	Vehicle Ctrl	5.88
1751-1770	Urethane	Positive Ctrl	6.48
1771-1790	1-9/84	1/2 X	6.04
1791-1810	2-9/84	1/2 X	5.90
1811-1830	3-9/84	1/2 X	6.06
1831-1850	4-9/84	1/2 X	6.07
1851-1870	5-9/84	1/2 X	5.81
1871-1890	1-9/84	1 X	5.86
1891-1910	2-9/84	1 X	5.48
1911-1930	3-9/84	1 X	5.56
1931-1950	4-9/84	1 X	5.46
1951-1970	5-9/84	1 X	5.78

Table 6-36 Mortality Data (Males)

Animal Nos.	Sample No.	Concentration Level	Dead
1451-1470	----	Untreated Ctrl	0
1471-1490	2% Emulphor	Vehicle Ctrl	1C
1491-1510	Urethane	Positive Ctrl	0
1511-1530	1-9/84	1/2 X	0
1531-1550	2-9/84	1/2 X	0
1551-1570	3-9/84	1/2 X	1C
1571-1590	4-9/84	1/2 X	0
1591-1610	5-9/84	1/2 X	1C
1611-1630	1-9/84	1 X	1A
1631-1650	2-9/84	1 X	0
1651-1670	3-9/84	1 X	0
1671-1690	4-9/84	1 X	0
1691-1710	5-9/84	1 X	1C

Table 6-37 Mortality Data (Females)

Animal Nos.	Sample No.	Concentration Level	Dead
1711-1730	----	Untreated Ctrl	0
1731-1750	2% Emulphor	Vehicle Ctrl	2C
1751-1770	Urethane	Positive Ctrl	0
1771-1790	1-9/84	1/2 X	1A
1791-1810	2-9/84	1/2 X	0
1811-1830	3-9/84	1/2 X	0
1831-1850	4-9/84	1/2 X	0
1851-1870	5-9/84	1/2 X	0
1871-1890	1-9/84	1 X	1E
1891-1910	2-9/84	1 X	0
1911-1930	3-9/84	1 X	0
1931-1950	4-9/84	1 X	0
1951-1970	5-9/84	1 X	1A

A = Died from gavage trauma
C = Died from unknown causes
E = Died due to holding error

Table 6-38 Percent Tumor-Bearing Animals (Males): Reconciled Count

Animal Nos.	Sample No.	Concentration Level	% TBA
1451-1470	----	Untreated Ctrl	15.0
1471-1490	2% Emulphor	Vehicle Ctrl	21.1
1491-1510	Urethane	Positive Ctrl	30.0
1511-1530	1-9/84	1/2 X	15.0
1531-1550	2-9/84	1/2 X	20.0
1551-1570	3-9/84	1/2 X	10.5
1571-1590	4-9/84	1/2 X	10.0
1591-1610	5-9/84	1/2 X	35.0
1611-1630	1-9/84	1 X	20.0
1631-1650	2-9/84	1 X	10.0
1651-1670	3-9/84	1 X	20.0
1671-1690	4-9/84	1 X	20.0
1691-1710	5-9/84	1 X	10.0

Table 6-39 Percent Tumor-Bearing Animals (Females): Reconciled Count

Animal Nos.	Sample No.	Concentration Level	% TBA
1711-1730	----	Untreated Ctrl	20.0
1731-1750	2% Emulphor	Vehicle Ctrl	5.6
1751-1770	Urethane	Positive Ctrl	35.0
1771-1790	1-9/84	1/2 X	5.2
1791-1810	2-9/84	1/2 X	5.3
1811-1830	3-9/84	1/2 X	0.0
1831-1850	4-9/84	1/2 X	0.0
1851-1870	5-9/84	1/2 X	15.0
1871-1890	1-9/84	1 X	15.8
1891-1910	2-9/84	1 X	25.0
1911-1930	3-9/84	1 X	25.0
1931-1950	4-9/84	1 X	0.0
1951-1970	5-9/84	1 X	0.0

TBA = Tumor-Bearing Animals

Table 6-40 Cumulative Nodules per Animal (Males): Reconciled Count

Animal Nos.	Sample No.	Concentration Level	Mean ±SD
1451-1470	----	Untreated Ctrl	0.20 ± 0.52
1471-1490	2% Emulphor	Vehicle Ctrl	0.42 ± 1.02
1491-1510	Urethane	Positive Ctrl	0.40 ± 0.75
1511-1530	1-9/84	1/2 X	0.20 ± 0.52
1531-1550	2-9/84	1/2 X	0.15 ± 0.37
1551-1570	3-9/84	1/2 X	0.11 ± 0.32
1571-1590	4-9/84	1/2 X	0.10 ± 0.31
1591-1610	5-9/84	1/2 X	0.35 ± 0.75
1611-1630	1-9/84	1/2 X	0.21 ± 0.42
1631-1650	2-9/84	1	0.15 ± 0.49
1651-1670	3-9/84	1	0.25 ± 0.55
1671-1690	4-9/84	1	0.20 ± 0.41
1691-1710	5-9/84	1	0.10 ± 0.31

Table 6-41 Cumulative Nodules Per Animal (Females): Reconciled Count

Animal Nos.	Sample No.	Concentration Level	Mean ± SD
1711-1730	----	Untreated Ctrl	0.20 ± 0.41
1731-1750	2% Emulphor	Vehicle Ctrl	0.06 ± 0.24
1751-1770	Urethane	Positive Ctrl	0.40 ± 0.60
1771-1790	1-9/84	1/2 X	0.05 ± 0.23
1791-1810	2-9/84	1/2 X	0.05 ± 0.22
1811-1830	3-9/84	1/2 X	0.00 ± 0.00
1831-1850	4-9/84	1/2 X	0.00 ± 0.00
1851-1870	5-9/84	1/2 X	0.15 ± 0.37
1871-1890	1-9/84	1 X	0.21 ± 0.54
1891-1910	2-9/84	1 X	0.30 ± 0.73
1911-1930	3-9/84	1 X	0.25 ± 0.44
1931-1950	4-9/84	1 X	0.00 ± 0.00
1951-1970	5-9/84	1 X	0.00 ± 0.00

SD = Standard Deviation

Table 6-42 Correlation of Gross Counts with Histopathology Findings

Animal No.	Observer/ Gross Counts 1	2	3[a]	Confirmed by Histopathology	% Correlation[b]
1457	1	1	1	1	100%
1498	1	1	1	1	100%
1525	0	1	1	1	100%
1600	0	1	1	0	0%
1676	2	1	1	1	100%
1686	1	1	1	1	100%
1761	1	1	1	1	100%
1867	1	1	1	1	100%
1898	1	1	1	1	100%
1910	1	0	0	0	100%
TOTALS	9	9	9	8	89%

[a] Reconciled count

[b] Correlation with with histopathology was based upon the reconciled gross count

APPENDIX A

DEVIATIONS FROM MODIFIED CONTRACT STATEMENT OF WORK: EPA Contract 68-03-1840

1. In Vivo Toxicity Test

a) Cage-side observations were performed twice a day.
Detailed observations were performed once a week.

b) Study 2 sample was administered by gavage, 0.3 ml per mouse.

2. Salmonella Typhimurium Mutagenesis Assay

a) 10/25/84 assay - S9 mix with 10% S9 concentration was used, 400 ul per plate.

b) 7/31/84 and 9/12/84 assays - 2-nitrofluorene doses were 1 ug and 2 ug.

c) 3/30/85 assay - samples were tested without S9 addition (except sample 27).

d) 9/12/84, 3/30/85, 5/14/85 assays - samples were tested without S9 addition.

e) Eighteen-sample series was tested at times 0 and 34 weeks.

f) Nine-sample series was tested at times 0, 1 week, 11 weeks, 33 weeks, and 62 weeks.

g) Top agar was prepared and stored, until used, in 2-ml aliquots with histidine-biotin solution already added.

f) Pre-mixed solutions of bacteria -S9 mix and bacteria-buffer were prepared just prior to experiment initiation and added to top agar tubes.

3. Rat Liver Focus Assay

a) Range of animal body weights at time of hepatectomies exceeded the specified range.

b) After hepatectomies, surgical silk was used for suturing and alcohol for disinfecting the incision site.

c) Promoter treatment began 7-9 days after the partial hepatectomies.

d) Animals were sacrificed by carbon dioxide inhalation.

e) Frozen tissue blocks were stored at -70°C.

f) Tissue sections were picked up on gelatin coated "subbed slides."

g) In the GGTase staining procedure, (1) the Fast Blue BB salt was mixed with 20 ml of incubation solution, (2) the incubation solution was more concentrated than specified and (3) the glycerin mounting medium used phenol instead of merthiolate.

4. Lung Adenoma Assay

The following deviations occurred in the first study (ETOX-84-1001-02):

a) For those animals found dead, a gross necropsy and lung examination was conducted on all, except for those animals that precluded pathological examination due to severe post mortem autolysis. Gross necropsy findings were entered in a necropsy log book and not on individual sheets. An entry of "DEAD" in this log was indicative of severe post mortem autolysis. No lung adenomas were grossly observed during the necropsy of animals found dead.

b) Photographs were taken of lung adenomas of positive control females during gross necropsy. These photographs were processed and when reviewed the adenomas were not identifiable. Photographs were not taken of the remaining animals.

c) Ten percent of the lungs were randomly selected and histopathologically evaluated for confirmation as lung adenomas by a qualified veterinary pathologist. The protocol stated all lungs would be sent for evaluation. (This was an error in the technical protocol, Section E, Necropsy). Upon technical review, additional lungs with adenomas were sent for evaluation.

STANDARD FORM 30, JULY 1966
GENERAL SERVICES ADMINISTRATION
FED. PROC. REG. (41 CFR) 1-16.101

AMENDMENT OF SOLICITATION/MODIFICATION OF CONTRACT

PAGE 1 OF 2

1. AMENDMENT/MODIFICATION NO. Two (2)

2. EFFECTIVE DATE

3. REQUISITION/PURCHASE REQUEST NO.

4. PROJECT NO. *(If applicable)*

5. ISSUED BY

Contracts Management Division
Environmental Protection Agency
Cincinnati, Ohio 45268

6. ADMINISTERED BY *(If other than block 5)* CODE

7. CONTRACTOR NAME AND ADDRESS CODE FACILITY CODE
(Street, city, county, state, and ZIP Code)

U.S. Small Business Administration
85 Marconi Boulevard
Columbus, OH 43215
Environmental Health Research & Testing, Inc.
3235 Omni Drive
Cincinnati, Ohio 45244

8.
☐ AMENDMENT OF SOLICITATION NO. ______
DATED ______ *(See block 9)*
☒ MODIFICATION OF CONTRACT/ORDER NO. 68-03-1840
(SB 58320396)
DATED 9/29/83 *(See block 11)*

9. THIS BLOCK APPLIES ONLY TO AMENDMENTS OF SOLICITATIONS

☐ The above numbered solicitation is amended as set forth in block 12. The hour and date specified for receipt of Offers ☐ is extended. ☐ is not extended.

Offerors must acknowledge receipt of this amendment prior to the hour and date specified in the solicitation, or as amended, by one of the following methods:

(a) By signing and returning ______ copies of this amendment; (b) By acknowledging receipt of this amendment on each copy of the offer submitted; or (c) By separate letter or telegram which includes a reference to the solicitation and amendment numbers. FAILURE OF YOUR ACKOWLEDGMENT TO BE RECEIVED AT THE ISSUING OFFICE PRIOR TO THE HOUR AND DATE SPECIFIED MAY RESULT IN REJECTION OF YOUR OFFER. If, by virtue of this amendment you desire to change an offer already submitted, such change may be made by telegram or letter, provided such telegram or letter makes reference to the solicitation and this amendment, and is received prior to the opening hour and date specified.

10. ACCOUNTING AND APPROPRIATION DATA *(If required)*

N/A

11. THIS BLOCK APPLIES ONLY TO MODIFICATIONS OF CONTRACTS/ORDERS

(a) ☐ This Change Order is issued pursuant to ______
The Changes set forth in block 12 are made to the above numbered contract/order.

(b) ☐ The above numbered contract/order is modified to reflect the administrative changes (such as changes in paying office, appropriation data, etc.) set forth in block 12.

(c) ☒ This Supplemental Agreement is entered into pursuant to authority of General Provisions, Clause 29, "Changes"
It modifies the above numbered contract as set forth in block 12.

12. DESCRIPTION OF AMENDMENT/MODIFICATION

Exhibit A to this Modification No. 2 delineates changes which are hereby incorporated into the Statement of Work under Contract No. 68-03-1840 and SBA subcontract no. 58320396. SBA, EPA, and the 8(a) subcontractor agree that these changes are made at no increase in the price or performance period of contract 68-03-1840 and subcontract 58320396. All other contract terms and conditions remain unchanged.

Attachment: Exhibit A - 4 pages.

Except as provided herein, all terms and conditions of the document referenced in block 8, as heretofore changed, remain unchanged and in full force and effect.

13. ☐ CONTRACTOR/OFFEROR IS NOT REQUIRED TO SIGN THIS DOCUMENT ☒ CONTRACTOR/OFFEROR IS REQUIRED TO SIGN THIS DOCUMENT AND RETURN (3) COPIES TO ISSUING OFFICE

14. NAME OF CONTRACTOR/OFFEROR
BY ______
(Signature of person authorized to sign)

17. UNITED STATES OF AMERICA
BY ______
(Signature of Contracting Officer)

15. NAME AND TITLE OF SIGNER *(Type or print)*
SEE PAGE TWO FOR SIGNATURES

16. DATE SIGNED

18. NAME OF CONTRACTING OFFICER *(Type or print)*
* SEE PAGE TWO FOR SIGNATURES

19. DATE SIGNED

U.S. GOVERNMENT PRINTING OFFICE: 1970-370-[illegible]

CONTRACT NO. 68-03-1840, Mod. No.2	SPECIAL PROVISIONS	PAGE 2 OF 2 PAGES

U.S. Small Business Administration

By: ______________________________ Date: ______________________________

Environmental Health Research & Testing, Inc.

By: P. S. Sabharwal Date: July 9, 1984

U.S. Environmental Protection Agency

By: ______________________________ Date: ______________________________
W.J. Bailey, Contracting Officer

Exhibit A
Mod. # 2
Contract 68-03-1840
Page 1 of 4

Changes in Contract Statement of Work Since Inception

Ames Assay

Item	Statement of of Work	Change Date	Changes Made
1. Amt. added/ Ames plate	0.05 to 0.4 ml	1-19-84 (verbal)	100 µl/plate of diluted samples
2. Solvent	Not specified; DMSO used in reference articles	1-12-84 (letter)	Emulphor
3. Positive Controls	2-anthramine (2 µg/plate)	1-19-84 (verbal)	2 µg/plate 1 µg/plate
	2-nitrofluorene (2 µg/plate)		5 µg/plate 1 µg/plate
	Na azide (none)		2 µg/plate 1 µg/plate
4. S-9 mix	According to Ames Mut. Res. 31:347, 1975	1-19-84 (verbal)	5% S9 in mix
5. Acetone samples	Concentrate acetone samples to 20% of original volume	1-19-84 (verbal)	Remove 150 ml to be stored at −20°C
6. Sample stability	Test at time 0, 1 wk, 1 month, 4 months, and 12 months	1-19-84 (verbal)	Eliminate 1 month test, do at 2 months Concentrate 50 ml of acetone sample at −20°C test at 2 months along with emulphor sample at 4°C
7. S9 mix	Testing with and without S9	3-22-84 (telephone)	Eliminate S9 mix in 2 month stability study
8. Doses	See Item #1	3-22-84 (telephone)	Increase top dose to 200 µl/ plate; 400 µl/ plate on sample #3

Exhibit A
Mod. #2
Contract 68-03-1840
Page 3 of 4

Ames Assay (Continuted)

Item	Statement of of Work	Change Date	Changes Made
9. Test samples	See Item #6	3-22-84 (telephone)	Test samples stored at 4C in emulphor and freshly concentrated from acetone
10. Doses	See Items #1, #8	3-28-84 (telephone)	Use 400 µl/ plate on all samples
11. Doses	See Items #1, #8, #9	3-28-84 (telephone)	Use 200 µl/ plate as top dose; 400 µl/ plate only on sample #3

EXHIBIT A
MOD. # 2
CONTRACT 68-03-1840
Page 3 of 4

Lung Adenoma Assay

Item	Statement of of Work	Change Date	Changes Made
1. Dose	0.5 ml/mouse 3x/wk8 wks	5-9-84 (telephone)	Dilute sample #6 1:2 Gavage animals with 0.25 ml/mouse

EXHIBIT A
MOD. #2
CONTRACT 68-03-1840
PAGE 4 OF 4

Sencar Assay

Item	Statement of of Work	Change Date	Changes Made
1. Dose	0.5 ml/mouse 3x/wk/2 wks	5-9-84 (telephone)	Dilute sample #6 1:2

Recd 1/24/84
HC

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
OFFICE OF RESEARCH AND DEVELOPMENT
HEALTH EFFECTS RESEARCH LABORATORY
CINCINNATI, OHIO 45268

January 12, 1984

Environmental Health Research and Testing Inc.
3235 Omni Drive
Cincinnati, OH 43215

Dear Sir:

This letter is to inform you the contractor that eight water samples for the test #1 In vivo Toxicity Test in Mice and the nine acetone samples for test #3 Sencar Mouse Skin Bioassay, #4 Rat Liver Foci Assay, #5 Lung Adenoma Assay are to be obtained by you for testing at 26 W. St. Clair Street, Cincinnati, Ohio during the week of January 16-20, 1984.

Acetone samples for 62 of the minimum 91 Salmonella Typhimurium Mutagenesis Assays are also available. Except for test #2, Salmonella Typhimurium Mutagenesis Assay, this will satisfy the minimum order reuqirements of this contract #CI-83-0965.

Samples to be tested under the above mentioned contract are to be prepared prior to testing by the contractor for each of the specified tests in the following manner:

I Aqueous Samples

A. In Vivo Toxicity Test in Mice - All 8 water samples are to be used in this test as supplied by EPA. Each sample is to be tested at two concentrations. Three of the samples will be supplied to the contractor having been previously prepared into two different concentrations. The other 5 aqueous samples are to be diluted by the contractor with deionized water to 1/4 of their original concentration for the second concentrations of these five samples. All samples are now sterile and should be handled in such a way as to remain sterile as they are removed for testing from their original container so as not to contaminate the original sample supply.

II Acetone Samples

Acetone samples shall be rotor evaporated in warm water bath (45°C) until all acetone is removed. The remaining aqueous sample will be approximately 20% of the original solution. The sample is then diluted (within the rotor flask) with a sterile 10% emulphor at a ratio of one part emulphor to 4 parts sample. The sample is immediately used in the respective tests:

A. **Salmonella Typhimurium Mutageneses Assay - Emulphored sample will be used in each assay. The schedule of sample stability testing is as follows:**

Sample #	Time in Months				
	0	1	4	6	12
1-9	1-9	1-9	1-9	-	1-9
1-9	1-9[a]	-	-	-	-
10-27	10-27	-	-	10-27	

[a] = Duplicate retesting of samples 1-9

B. Sencar Mouse Bioassay - The emulphored sample will be dosed orally at 0.5 ml per mouse 3 times per week for 2 weeks for a total dose of 3.0 ml per mouse. There will be 9 samples to be tested.

C. Rat Liver Foci Assay - The emulphored sample will be dosed orally at 3.0 ml per rat. There will be 9 samples to be tested.

D. Lung Adenoma Assay - This test shall include two concnetrations of each emulphored sample. One concentration shall be as the original emulphored sample while the second concentration shall be 1/2 of the original. This is accomplished by diluting an aliquot of the original sample with 2% sterile emulphor. Each emulphored test sample will be dosed orally at 0.5 ml per mouse three times per week for eight weeks for a total of 12.0 ml per mouse. A total of 9 samples are to be tested; each at two different concentrations.

The volume of each of the 9 acetone samples to be furnished the contractor for preparation prior to testing will be 5.0 liters. Acetone samples for only Ames Testing will have a volume of 80 ml.

The aqueous samples which require no pre-treatment will be supplied at a volume of 6.0 liters.

We expect EHRT (contractor) to obtain these samples and immediately proceed with sample prepartion, animal ordering and testing to meet the terms of the above mentioned contract.

Sincerely yours,

Robert G. Miller

Robert G. Miller, Project Officer
Chemical and Statistical Support Branch
Toxicology and Microbiology Division

cc: Bill Wise, Contract Officer

APPENDIX B

QUALITY ASSURANCE STATEMENTS

QUALITY ASSURANCE STATEMENT

TITLE: In Vivo Toxicity Testing of Concentrated Drinking and Waste Waters

SPONSOR: Environmental Protection Agency

PROJECT NUMBER: 68-03-1840

STUDY DATES: February 7, 1984 through March 24, 1984

STUDY DIRECTOR: Dr. H. Choudhury

Virus and parasite free CD-1 mice from Harlan Industries, Inc., Indianapolis, IN, were immediately placed in quarantine. They were inspected for general health and suitability as test animals.

Water samples were provided by the EPA project officer. Aliquots from samples 1 through 5 were diluted (1/4) at EHRT for the second dose, where as samples 6 through 8 were provided by the EPA at both 1X and 10X concentrations.

The quality assurance schedule of operations is presented on the following pages.

Arlene F Kingery

Arlene F. Kingery, Director
Quality Assurance

STANDARD STATEMENT PARAGRAPH

	Date(s) of Inspection	Date(s) Reported to Study Director	Date(s) Reported to Management
Protocol	1-23-84 1-24-84	3-23-84	4-5-84
Body Weight Data	3-23-84	3-23-84	4-5-84
Water Consumption Data	3-23-84	3-23-84	4-5-84
Animal Records	3-23-84	3-23-84	4-5-84
Necropsy			
Organ Weights	3-23-84	3-23-84	4-5-84
Gross Findings	3-23-84	3-23-84	4-5-84

The observations, study records, and final reports of this study were inspected in accordance with Good Laboratory Practice Regulations, the Standard Operating Procedure of EHRT Laboratories, Inc., and the protocol. The study was conducted in compliance with GLP's, SOP's and the sponsor's protocol. To the best of the signatory's knowledge, this final report accurately describes the study procedure used and accurately reflects the raw data. There were no significant deviations from GLP's which would affect the quality or integrity of the study. The Quality Assurance findings, derived from the inspections are documented and have been made available to the study director and to the test facility management.

Arlene F Kingery

Arlene F. Kingery, Director
Quality Assurance

The raw data and other materials, as well as a copy of the final and interim reports, will be kept in archives at EHRT laboratories, Inc.

April 4, 1984
Date

Arlene F Kingery
Arlene F. Kingery, Director
Quality Assurance

QUALITY ASSURANCE STATEMENT

TITLE: In Vivo Toxicity Testing of Concentrated Drinking and Waste Waters

SPONSOR: U.S. Environmental Protection Agency

PROJECT NUMBER: 68-03-1840

STUDY DATES: October 16, 1984 through November 30, 1984

STUDY DIRECTOR: Dr. H. Choudhury

Virus and parasite free CD-1 mice from Harlan Industries, Inc., Indianapolis, IN., were immediately placed in quarantine. They were inspected for general health and suitability as test animals.

Concentrated Water samples were provided by the EPA project officer. Aliquots from samples 1 through 5 were diluted (1/4) at E.H.R.T. for the second dose.

The quality assurance schedule of operations is presented on the following pages.

Arlene F. Kingery, Director
Quality Assurance

STANDARD STATEMENT PARAGRAPH

	DATE(S) OF INSPECTION	DATE(S) REPORTED TO STUDY DIRECTOR	DATE(S) REPORTED TO MANAGEMENT
PROTOCOL	1-23-84 1-24-84	3-23-84	4-5-84
BODY WEIGHT DATA	12-10-84	3-23-84	4-5-84
ANIMAL WEIGHING AND DOSING (GAVAGE) OBSERVATIONS	11-12-84	11-12-84	12-10-84
ANIMAL RECORDS	11-12-84	3-23-84	4-5-84
NECROPSY			
ORGAN WEIGHTS	11-12-84	11-12-84	12-10-84
GROSS FINDINGS	11-12-84	11-12-84	12-10-84

The observations, study records, and final reports of this study were inspected in accordance with Good Laboratory Practice Regulations, the Standard Operating Procedure of E.H.R.T. Laboratories, Inc., and the protocol. The study was conducted in compliance with GLP's, SOP's and the sponsor's protocol. To the best of the signatory's knowledge, this final report accurately describes the study procedure used and accurately reflects the raw data. There were no significant deviations from GLP's which would affect the quality or integrity of the study. The Quality Assurance findings, derived from the inspections are documented and have been made available to the study director and to the test facility management.

Arlene F. Kingery, Director
Quality Assurance

The raw data and other materials, as well as a copy of the final and interim reports, will be kept in archives at E.H.R.T. Laboratories, Inc..

Dec 10, 1984
Date

Arlene F. Kingery
Arlene F. Kingery, Director
Quality Assurance

Quality Assurance Statement

Title: Mutagenicity Testing of Waste Water Organic Acetone Samples in the Ames Assay

Sponsor: U.S. Environmental Protection Agency

Contract No.. 68-03-1840

Assay Dates:		
	2-02-84	9-12-84
	2-09-84	10-25-84
	4-06-84	3-30-85
	7-31-84	5-14-85

Study Director: Tom Hughes, RTI

Program Director: Dr. Bruce Casto, EHRT

Schedule of Inspections

Phase Inspected	Date(s) of Inspection	Date(s) Reported to Program Director
Protocol review	1-17-84	1-18-84
Observation of testing procedures - sample dilution - S9 mix preparation - plate incorporation procedure	2-02-84	2-02-84
Observation of testing procedures - counting of test plates	2-07-84	2-07-84
System review - general conduct - SOP's - equipment - test and control articles	4-02-84	4-02-84

Internal quality control data (generated with each experiment) - positive controls - sterility checks - bacterial strain checks	2-22-84	2-22-84
	4-23-84	4-23-84
	8-06-84	8-06-84
	10-03-84	10-03-84
	11-26-84	11-26-84
	4-10-85	4-10-85
	5-16-85	5-16-85

The protocols, laboratory procedures, and assay reports of this study were inspected in compliance with Good Laboratory Practice Regulations. To the best of my knowledge, the assay reports accurately describe the study procedure used and accurately reflect the raw data. The Quality Assurance findings derived from the inspections are documented and have been made available to the Program Director.

Copies of the raw data and the Assay Reports will be kept in archives at EHRT, R.T.P., N.C. All of the raw data and the original Assay Reports will be retained in the archives at Research Triangle Institute, R.T.P., N.C.

Linda Snow

Linda Snow
Quality Assurance Specialist, EHRT

Dec. 16, 1985

Date

QUALITY ASSURANCE STATEMENT

Study Title: SENCAR MOUSE SKIN BIOASSAY FOR DETERMINING THE INITIATING POTENTIAL OF CONCENTRATED DRINKING OR WASTE WATER SAMPLES

Study Number: 1840.01

Study Director: M. WENK, PH.D.

Initiation Date: MAY 21, 1984

Review Completed Date: SEPTEMBER 27, 1985

This study has been divided into a series of phases. Quality Assurance monitors each of these phases periodically over the course of the study. Procedures, documentation, equipment, etc. are examined in order to assure the the study is performed in accordance with the Good Laboratory Practice regulations and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

DATES/PHASES

INSPECT ON 84/04/25 - 84/04/25,TO STUDY DIR 84/04/25,TO MGMT 84/04/25

PHASES: PROTOCOL REVIEW

INSPECT ON 84/05/14 - 84/05/14,TO STUDY DIR 84/05/14,TO MGMT 84/05/17

PHASES: RANDOMIZATION
QUARANTINE/ALLOCATION TO HOUSING

INSPECT ON 84/05/16 - 84/05/16,TO STUDY DIR 84/05/17,TO MGMT 84/05/24

PHASES: DOSE PREPARATION

INSPECT ON 84/05/21 - 84/05/21,TO STUDY DIR 84/05/21,TO MGMT 84/05/24

PHASES: DOSING

INSPECT ON 84/06/13 - 84/06/13,TO STUDY DIR 84/06/15,TO MGMT 84/06/21

PHASES: TPA DOSE PREPARATION

INSPECT ON 84/08/13 - 84/08/13,TO STUDY DIR 84/08/15,TO MGMT 84/08/24

PHASES: PROMOTION

INSPECT ON 84/09/19 - 84/09/19,TO STUDY DIR 84/09/24,TO MGMT 84/10/01

PHASES: TPA DOSE PREPARATION

INSPECT ON 84/10/22 - 84/10/22,TO STUDY DIR 84/10/22,TO MGMT 84/10/24

PHASES: BODY WEIGHTS

INSPECT ON 84/12/20 - 84/12/20,TO STUDY DIR 84/12/20,TO MGMT 85/01/07

PHASES: NECROPSY

INSPECT ON 85/07/26 - 85/08/19,TO STUDY DIR 85/08/19,TO MGMT 85/09/27

PHASES: DRAFT REPORT

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Nona S Karten 9/27/85

Quality Assurance
RA/QA Department

Date

STATEMENT OF COMPLIANCE

To the best of my knowledge, Study 1840.01 (SENCAR Mouse Skin Bioassay for Determining the Initiating Potential of Concentrated Drinking or Waste Water Samples) was conducted in compliance with the Good Laboratory Practices regulations as published in 21 CFR 58, 40 CFR 160 and 40 CFR 792 in all material aspects with the following reservations:

> The identity, strength, purity, and composition or other characteristics to define the test or control substances have not been determined by the testing facility (Section 105(a)).
>
> The stability of the test or control substances under the test conditions has not been determined by the testing facility and is not included in the final report (Sections 105 (a) and (b) and 185 (a)(5)).
>
> Analyses to determine the uniformity, concentration, or stability of the test or control mixtures were not performed by the testing facility (Section 113 (a)).

Signature

Study Director

Martin L. Hench

9/27/85
Date

QUALITY ASSURANCE STATEMENT

This histopathology project has been conducted in compliance with Good Laboratory Practices regulations promulgated by the U.S. Food and Drug Administration. Pathology Associates, Incorporated has a functioning and responsive quality assurance unit which has conducted appropriate inspections and reported all findings to management. The following is a record of those inspections and reports:

Date of Inspection	Phase Inspected	Date Findings Reported To Management and Study Director
March 29, 1985	Data	April 1, 1985
April 1, 1985	Draft Final Report	April 1, 1985
April 30, 1985	Data (add.)	April 30, 1985
May 1, 1985	2nd Draft Final Report	May 1, 1985
May 6, 1985	Final Report	May 6, 1985

Director, Quality Assurance Unit

05-06-85
Date

Study No. 1840.01 - Sencar Mice, Skin Bioassay

QUALITY ASSURANCE STATEMENT

Study Title: SENCAR MOUSE SKIN BIOASSAY FOR DETERMINING THE INITIATING POTENTIAL OF CONCENTRATED DRINKING OR WASTE WATER SAMPLES

Study Number: 1840.03

Study Director: Martin L. Wenk, Ph.D.

Initiation Date: August 13, 1984

Review Completed Date: September 30, 1985

This study has been divided into a series of phases. Quality Assurance monitors each of these phases periodically over the course of the study. Procedures, documentation, equipment, etc. are examined in order to assure the the study is performed in accordance with the Good Laboratory Practice regulations and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

DATES/PHASES

INSPECT ON 84/04/25 - 84/04/25,TO STUDY DIR 84/04/25,TO MGMT 84/04/25

PHASES: PROTOCOL REVIEW

INSPECT ON 84/06/13 - 84/06/13,TO STUDY DIR 84/06/15,TO MGMT 84/06/21

PHASES: TPA DOSE PREPARATION

INSPECT ON 84/06/25 - 84/06/25,TO STUDY DIR 84/06/27,TO MGMT 84/07/05

PHASES: RANDOMIZATION
ALLOCATION TO HOUSING

INSPECT ON 84/06/27 - 84/06/27,TO STUDY DIR 84/06/27,TO MGMT 84/06/28

PHASES: DOSE PREPARATION

INSPECT ON 84/08/06 - 84/08/06,TO STUDY DIR 84/08/07,TO MGMT 84/08/07

PHASES: RANDOMIZATION AND ANIMAL IDENTIFICATION

INSPECT ON 84/08/13 - 84/08/13,TO STUDY DIR 84/08/15,TO MGMT 84/08/24

PHASES: DOSING (INITIATION)

INSPECT ON 84/09/19 - 84/09/19,TO STUDY DIR 84/09/24,TO MGMT 84/10/01

PHASES: TPA DOSE PREPARATION

INSPECT ON 84/10/19 - 84/10/19,TO STUDY DIR 84/10/22,TO MGMT 84/10/24

PHASES: PROMOTION

INSPECT ON 84/10/23 - 84/10/23,TO STUDY DIR 84/10/23,TO MGMT 84/10/29

PHASES: BODY WEIGHTS

INSPECT ON 84/12/18 - 84/12/18,TO STUDY DIR 84/12/20,TO MGMT 85/01/07

PHASES: SKIN TUMOR DATA COLLECTION

INSPECT ON 85/03/11 - 85/03/11,TO STUDY DIR 85/03/12,TO MGMT 85/03/15

PHASES: NECROPSY

INSPECT ON 85/07/31 - 85/08/16,TO STUDY DIR 85/08/19,TO MGMT 85/09/27

PHASES: DRAFT REPORT

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Joseph 10/1/85

Quality Assurance
RA/QA Department

Date

STATEMENT OF COMPLIANCE

To the best of my knowledge, Study 1840.03 (SENCAR Mouse Skin Bioassay for Determining the Initiating Potential of Concentrated Drinking or Waste Water Samples) was conducted in compliance with the Good Laboratory Practices regulations as published in 21 CFR 58, 40 CFR 160 and 40 CFR 792 in all material aspects with the following reservations:

> The identity, strength, purity, and composition or other characteristics to define the test or control substances have not been determined by the testing facility (Section 105(a)).
>
> The stability of the test or control substances under the test conditions has not been determined by the testing facility and is not included in the final report (Sections 105 (a) and (b) and 185 (a)(5)).
>
> Analyses to determine the uniformity, concentration, or stability of the test or control mixtures were not performed by the testing facility (Section 113 (a)).

Signature

Study Director

Martin L Wenk

10/1/85
Date

QUALITY ASSURANCE STATEMENT

This histopathology project has been conducted in compliance with Good Laboratory Practices regulations promulgated by the U.S. Food and Drug Administration. Pathology Associates, Incorporated has a functioning and responsive quality assurance unit which has conducted appropriate inspections and reported all findings to management. The following is a record of those inspections and reports:

Date of Inspection	Phase Inspected	Date Findings Reported to Management and Study Director
April 09, 1985	Trimming	April 09, 1985
May 02, 1985	Data	May 02, 1985
May 10, 1985	Draft Final Report	May 10, 1985
May 15, 1985	Final Report	May 15, 1985

Director, Quality Assurance Unit

May 15, 1985

Date

Study No. 1840.03
Sencar Mice - Females, Skin Bioassay

QUALITY ASSURANCE STATEMENT

Study Title: SENCAR MOUSE SKIN BIOASSAY FOR DETERMINING
THE INITIATING POTENTIAL OF CONCENTRATED
DRINKING OR WASTE WATER SAMPLES

Study Number: 1840.04

Study Director: Martin L. Wenk, Ph.D.

Initiation Date: 84/08/27

Review Completed Date: 85/10/02

This study has been divided into a series of phases. Quality Assurance monitors each of these phases periodically over the course of the study. Procedures, documentation, equipment, etc. are examined in order to assure the the study is performed in accordance with the Good Laboratory Practice regulations and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

DATES/PHASES

INSPECT ON 84/05/10 - 84/05/10,TO STUDY DIR 84/05/10,TO MGMT 84/05/10

PHASES: PROTOCOL REVIEW

INSPECT ON 84/06/13 - 84/06/13,TO STUDY DIR 84/06/15,TO MGMT 84/06/21

PHASES: TPA DOSE PREPARATION

INSPECT ON 84/08/20 - 84/08/20,TO STUDY DIR 84/08/21,TO MGMT 84/09/05

PHASES: RANDOMIZATION
ALLOCATION TO HOUSING

INSPECT ON 84/08/22 - 84/08/22,TO STUDY DIR 84/08/29,TO MGMT 84/09/05

PHASES: DOSE PREPARATION

INSPECT ON 84/09/05 - 84/09/05,TO STUDY DIR 84/09/05,TO MGMT 84/09/07

PHASES: DOSING

INSPECT ON 84/09/19 - 84/09/19,TO STUDY DIR 84/09/24,TO MGMT 84/10/01

PHASES: TPA DOSE PREPARATION

INSPECT ON 84/10/19 - 84/10/19,TO STUDY DIR 84/10/22,TO MGMT 84/10/24

PHASES: PROMOTION

INSPECT ON 84/11/05 - 84/11/05,TO STUDY DIR 84/11/05,TO MGMT 84/11/07

PHASES: BODY WEIGHTS

INSPECT ON 84/11/15 - 84/11/15,TO STUDY DIR 84/11/21,TO MGMT 84/11/27

PHASES: TUMOR DATA COLLECTION

INSPECT ON 85/02/14 - 85/02/14,TO STUDY DIR 85/02/14,TO MGMT 85/02/18

PHASES: SKIN TUMOR DATA COLLECTION

INSPECT ON 85/03/27 - 85/03/27,TO STUDY DIR 85/03/29,TO MGMT 85/04/04

PHASES: NECROPSY

INSPECT ON 85/07/30 - 85/08/13,TO STUDY DIR 85/08/13,TO MGMT 85/10/02

PHASES: DRAFT REPORT

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Joseph Appellan　　10/3/85

Quality Assurance　　Date
RA/QA Department

STATEMENT OF COMPLIANCE

To the best of my knowledge, Study 1840.04 (SENCAR Mouse Skin Bioassay for Determining the Initiating Potential of Concentrated Drinking or Waste Water Samples) was conducted in compliance with the Good Laboratory Practices regulations as published in 21 CFR 58, 40 CFR 160 and 40 CFR 792 in all material aspects with the following reservations:

> The identity, strength, purity, and composition or other characteristics to define the test or control substances have not been determined by the testing facility (Section 105(a)).
>
> The stability of the test or control substances under the test conditions has not been determined by the testing facility and is not included in the final report (Sections 105 (a) and (b) and 185 (a)(5)).
>
> Analyses to determine the uniformity, concentration, or stability of the test or control mixtures were not performed by the testing facility (Section 113 (a)).

Signature

Study Director

10/2/85
Date

QUALITY ASSURANCE STATEMENT

This histopathology project has been conducted in compliance with Good Laboratory Practices regulations promulgated by the U.S. Food and Drug Administration. Pathology Associates, Incorporated has a functioning and responsive quality assurance unit which has conducted appropriate inspections and reported all findings to management. The following is a record of those inspections and reports:

Date of Inspection	Phase Inspected	Date Findings Reported to Management and Study Director
June 20, 1985	Raw data and computer generated individual animal data sheets	June 20, 1985
June 27, 1985	Computer generated tables and draft pathology report	June 28, 1985
July 08, 1985	Final pathology report	July 08, 1985

Director, Quality Assurance Unit

July 08, 1985

Date

Study No. 1840.04 - SENCAR Mouse Skin Bioassay for Determining the Initiating Potential of Concentrated Drinking or Waste Water Samples

QUALITY ASSURANCE STATEMENT

Study Title: RAT LIVER FOCUS ASSAY FOR DETERMINING THE INITIATING POTENTIAL OF CONCENTRATEED DRINKING OR WASTE WATER SAMPLES

Study Number: 1840.02

Study Director: MARTIN L. WENK, PH.D.

Initiation Date: 84/04/09

Review Completed Date: 85/09/06

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc., are examined in order to assure that the study is performed in accordance with the Good Laboratory Practice regulations and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

DATES/PHASES

INSPECT ON 84/03/30 - 84/03/30,TO STUDY DIR 84/03/30,TO MGMT 84/03/30

PHASES: PROTOCOL REVIEW

INSPECT ON 84/04/11 - 84/04/11,TO STUDY DIR 84/04/12,TO MGMT 84/04/13

PHASES: PARTIAL HEPATECTOMY

INSPECT ON 84/04/12 - 84/04/12,TO STUDY DIR 84/04/12,TO MGMT 84/04/13

PHASES: DOSING

INSPECT ON 85/04/30 - 85/05/06,TO STUDY DIR 85/05/06,TO MGMT 85/05/15

PHASES: DRAFT REPORT

INSPECT ON 85/09/04 - 85/09/04,TO STUDY DIR 85/09/04,TO MGMT 85/09/06

PHASES: DRAFT TO FINAL REPORT

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Norma S Karten 9/6/85

Quality Assurance Date
RA/QA Department

STATEMENT OF COMPLIANCE

To the best of my knowledge, Study 1840.02 (Rat Liver Focus Assay for Determining the Initiating Potential of Concentrated Drinking or Waste Water Samples) was conducted in compliance with the Good Laboratory Practices regulations as published in 21 CFR 58, 40 CFR 160 and 40 CFR 792 in all material aspects with the following reservations:

> The identity, strength, purity, and composition or other characteristics to define the test and control substances have not been determined by the testing facility (Section 105(a)).
>
> The stability of the test and control substances under the test conditions has not been determined by the testing facility and is not included in the final report (Sections 105 (a) and (b) and 185 (a)(5)).
>
> Analyses to determine the uniformity, concentration, or stability of the test and control mixtures were not performed by the testing facility (Section 113 (a)).

Signature

Study Director

9/6/85

Date

QUALITY ASSURANCE STATEMENT

This histopathology project has been conducted in compliance with Good Laboratory Practices regulations promulgated by the U.S. Food and Drug Administration. Pathology Associates, Incorporated has a functioning and responsive quality assurance unit which has conducted appropriate inspections and reported all findings to management. The following is a record of those inspections and reports:

Date of Inspection	Phase Inspected	Date Findings Reported To Management and Study Director
January 28, 1985	Final Report	January 28, 1985
March 24, 1985	Amended Final Report	March 25, 1985

James D Stevens 03-25-85

Director, Quality Assurance Unit

Study No. 1840.02 - Rat Liver Focus Assay

QUALITY ASSURANCE STATEMENT *

This histopathology project has been conducted in compliance with Good Laboratory Practices regulations promulgated by the U.S. Food and Drug Administration. Pathology Associates, Incorporated has a functioning and responsive quality assurance unit which has conducted appropriate inspections and reported all findings to management. The following is a record of those inspections and reports:

Date of Inspection	Phase Inspected	Date Findings Reported to Management and Study Director
November 07, 1984	GGTase Enzyme Staining	November 07, 1984
November 07, 1984	Frozen Microtomy	November 07, 1984
January 28, 1985	Data	January 28, 1985

Director, Quality Assurance Unit

August 23, 1985

Date

Study No. 1840.02 - Rat Liver Focus Assay

*This Quality Assurance Statement is being supplied to supplement the original statement in which the above noted phase inspections were inadvertently omitted.

QUALITY ASSURANCE STATEMENT

Study Title: RAT LIVER FOCUS ASSAY FOR DETERMINING THE INITIATING POTENTIAL OF CONCENTRATED DRINKING OR WASTE WATER SAMPLES

Study Number: 1840.05

Study Director: Martin L. Wenk, Ph.D.

Initiation Date: 11/7/84

Review Completed Date: 9/6/85

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc., are examined in order to assure that the study is performed in accordance with the Good Laboratory Practice regulations and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

DATES/PHASES

INSPECT ON 84/10/22 - 84/10/22,TO STUDY DIR 84/10/22,TO MGMT 84/10/22

PHASES: PROTOCOL REVIEW

INSPECT ON 84/11/13 - 84/11/15,TO STUDY DIR 84/11/21,TO MGMT 84/11/27

PHASES: PROMOTER PREPARATION

INSPECT ON 85/01/16 - 85/01/16,TO STUDY DIR 85/01/18,TO MGMT 85/01/22

PHASES: LIVER TISSUE COLLECTION

INSPECT ON 85/05/20 - 85/05/21,TO STUDY DIR 85/05/28,TO MGMT 85/05/28

PHASES: DRAFT REPORT

INSPECT ON 85/09/04 - 85/09/04,TO STUDY DIR 85/09/05,TO MGMT 85/09/06

PHASES: DRAFT TO FINAL REPORT

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Joseph Oppelbaum 9/6/85

Quality Assurance Date
RA/QA Department

STATEMENT OF COMPLIANCE

To the best of my knowledge, Study 1840.05 (Rat Liver Focus Assay for Determining the Initiating Potential of Concentrated Drinking or Waste Water Samples) was conducted in compliance with the Good Laboratory Practices regulations as published in 21 CFR 58, 40 CFR 160 and 40 CFR 792 in all material aspects with the following reservations:

> **The identity, strength, purity, and composition or other characteristics to define the test and control substances have not been determined by the testing facility (Section 105(a)).**
>
> The stability of the test and control substances under the test conditions has not been determined by the testing facility and is not included in the final report (Sections 105 (a) and (b) and 185 (a)(5)).
>
> Analyses to determine the uniformity, concentration, or stability of the test and control mixtures were not performed by the testing facility (Section 113 (a)).

Signature

Study Director

Martin L. Wenk

9/6/85
Date

QUALITY ASSURANCE STATEMENT

This histopathology project has been conducted in compliance with Good Laboratory Practices regulations promulgated by the U.S. Food and Drug Administration. Pathology Associates, Incorporated has a functioning and responsive quality assurance unit which has conducted appropriate inspections and reported all findings to management. The following is a record of those inspections and reports:

Date of Inspection	Phase Inspected	Date Findings Reported To Management and Study Director
February 27, 1985	GGTase Enzyme Staining	February 27, 1985
February 27, 1985	Frozen Microtomy	February 27, 1985
April 29, 1985	Data	April 30, 1985
April 29, 1985	Preliminary Draft of Path. Report	April 30, 1985
May 16, 1985	Final Pathology Report	May 16, 1985

James D. Stevens	May 16, 1985
Director, Quality Assurance Unit	Date

Study Number 1840.05 - Rat Liver Focus Assay for Determining the Initiating Potential of Concentrated Drinking or Waste Water Samples.

QUALITY ASSURANCE STATEMENT

TITLE: Mice Lung Adenoma Assay for Determining the Initiating Potential of Concentrated Drinking or Waste Water Samples

SPONSOR: U.S. Environmental Protection Agency

STUDY NUMBER:

ETOX-84-1001-02

PROJECT NUMBER:

68-03-1840

STUDY DATES:

April 30, 1984 through November 13, 1984

SCIENTIFIC DIRECTOR:

Dr. Bruce Casto, Sc.D.

SCHEDULE OF OPERATIONS:

	DATE(S) OF INSPECTION	DATE(S) REPORTED TO STUDY DIRECTOR	DATE(S) REPORTED TO MANAGEMENT
Protocol	04/28/84	04/28/84	04/28/84
Body Weight Data	12/10/84	12/10/84	12/10/84
Animal Records	11/12/84 12/10/84	11/12/84 12/10/84	11/12/84 12/10/84
Necropsy Gross Findings	11/12/84	11/12/84	11/12/84
Lung Adenoma Counts	11/12/84	11/12/84	11/12/84

The observations, study records, and final reports of this study were inspected in accordance with Good Laboratory Practice Regulations, the Standard Operating Procedure of E.H.R.T. Laboratories, Inc., and the protocol. The study was conducted in compliance with GLP's, SOP's and the sponsor's protocol. To the best of the signatory's knowledge, this final report accurately describes the study procedure used and accurately reflects the raw data. There were no significant deviations from GLP's which would affect the quality or integrity of the study. The Quality Assurance findings, derived from the inspections are documented and have been made available to the study director and to the test facility managment.

The raw data and other materials, as well as a copy of the final and interim reports, will be kept in archives at E.H.R.T. Laboratories, Inc..

Arlene F Kingery	March 4, 1985
Arlene Kingery Quality Assurance	DATE

ENVIRONMENTAL HEALTH RESEARCH AND TESTING, INC.
3235 OMNI DRIVE CINCINNATI, OHIO 45245

**ANALYTICAL TESTING
LABORATORY
(513) 752-2950**

April 1, 1985

Mr. Robert Miller
U.S. Environmental Protection Agency
26 W. St. Clair
Health Effects Research Laboratory
Cincinnati, Ohio 45268

Ref.: Study Number: ETOX-84-1001-2
Project Number: 68-03-1840
Title: Mice Lung Adenoma Assay for Determining the Initiating Potential of Concentrated Drinking or Waste Water Samples.

Dear Mr. Miller,

Enclosed please find a copy of the quality assurance report you inquired about. The inspection date in the final report for the Lung Adenoma Counts was incorrect. The report should read as follows:

	DATE OF INSPECTION	DATE REPORTED TO STUDY DIRECTOR	DATE REPORTED TO MANAGEMENT
Lung Adenoma Counts	12/10/84	12/10/84	12/10/84

I am sorry for any incovenience this may have caused you. If you have any questions, please feel free to call me at (606) 278-0603.

Sincerely,

Arlene F Kingery
Arlene Kingery
Quality Assurance Officer

AK/pb

Enclosure

QUALITY ASSURANCE STATEMENT

TITLE: Mouse Lung Adenoma Assay for Determining the Initiating Potential of Five Concentrated Drinking or Waste Water Samples

SPONSER: US Environmental Protection Agency

PROJECT NUMBER: ETOX-84-1001-04

STUDY DATES: December 18, 1984 through September 6, 1985

STUDY DIRECTOR: Dr. Raymond G. York

PROGRAM DIRECTOR: Dr. Bruce Casto

SCHEDULE OF INSPECTIONS

	DATE(S) OF INSPECTION	DATE(S) REPORTED TO STUDY DIRECTOR
Protocol	10/04/85	10/04/85
Body Weight Data	08/12/85 10/07/85	08/12/85 10/07/85
Animal Records	01/09/85	01/09/85
oral gavage	01/16/85	01/16/85
room book (log/study)	02/06/85	02/06/85
health surveillance	04/09/85	04/09/85
Study Log Book	10/07/85	10/07/85
Necropsy (gross)	06/11/85	06/11/85
Lung Nodule Counts	08/12/85	08/12/85

The observations, study records, and final reports of this study were inspected in accordance with Good Laboratory Practice Regulations. To the best of the signatory's knowledge, this final report accurately describes the study procedure used and accurately reflects the raw data. There were no significant deviations from the contract. The Quality Assurance finding, derived from the inspections, are documented and have been made available to the study director and to the test facility management.

The raw data and other materials, as well as a copy of the final and interim reports, will be kept in the archives at Environmental Health Research and Testing, Inc.

Willa Bailes

Willa Bailes
Quality Assurance Officer

10-31-85
Date

Quality Assurance Review of Final Report

The Final Report for EPA contract 68-03-1840 was reviewed. Data in the tables were consistent with the conclusions discussed in the text of the report. Deviations from the contract Statement of Work were summarized in Appendix A and described within the text of the Final Report under Materials and Methods or Experimental Procedures. QA review of the Final Report was documented and made available to the EHRT Program Director. Copies of this review and of the Final Report will be kept in the archives at EHRT, RTP, N.C.

Betty F. Hyatt　　　　2-20-86

Betty F. Hyatt　　　　Date

QA Officer, EHRT-RTP